**UNIVERSIDAD COMPLUTENSE DE MADRID**
FACULTAD DE VETERINARIA


# TESIS DOCTORAL

## Análisis epidemiológico y molecular de los principales patógenos en *Apis mellifera* y su importancia en el desencadenamiento del colapso de las colmenas

## Epidemiological and molecular analysis of the main pathogens of *Apis mellifera* and their importance in triggering colony losses

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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**UNIVERSIDAD COMPLUTENSE DE MADRID**

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Departamento de Sanidad Animal

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MEMORIA DE TESIS DOCTORAL

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estudiante en el Programa de Doctorado en Veterinaria,
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Análisis epidemiológico y molecular de los principales patógenos en Apis mellifera y su importancia en el desencadenamiento del colapso de las colmenas

y dirigida por: Prof. Joaquín Goyache Goñi y Prof. José Manuel Sánchez-Vizcaíno Rodríguez

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A Ohm, Lola y Bruno.

Sois el impulso que me motiva
para querer cambiar el mundo.

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SUAT tiene muchas casas, todas ellas habitadas por personas maravillosas. Por eso tengo que dar las gracias a todos los colegas del CISA, en especial a Matilde, por tu dulzura y cercanía. Espero que sigamos disfrutando juntas del zumbido de las abejas en futuros proyectos. También a Miguel, por tu determinación y amabilidad. A Marisa, Ana, Irene y Fernando. Y, cómo no, a mi querida Marti: gracias por tu positivismo, sabes hacer que germinen flores en los huecos que deja la tristeza. Gracias también a los compañeros de VISAVET, siempre dispuestos a echar una mano y sin los cuales aquella "Rave del fondo" en la fiesta de Navidad de 2016 no habría tenido sentido. Mención especial a Javi y Carlos, los médicos de ordenadores más majos del mundo, y a Irene, mi compañera de luchas durante la carrera (lograrás todo lo que te propongas, amiga).

Ahora pienso en otra casa a kilómetros de distancia. Evoco la sensación "get lost" y sonrío, transportándome de inmediato a Montana State University. Recuerdo el miedo, la incertidumbre y el desasosiego justo antes de coger el avión. He de confesar que estuve a punto de darme la vuelta en numerosas ocasiones justo antes de atravesar el control de seguridad, pero seguí adelante a pesar de las dudas y, gracias a ello, tuve la oportunidad de disfrutar una de las experiencias más enriquecedoras de mi vida. "Get lost", repito. A veces hay que irse lejos para estar más cerca de uno mismo. Gracias a todos los compañeros de aquellos meses, especialmente a Michelle, por toda tu ayuda y cariño, pero, sobre todo, por ser esa

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Claro que, a veces, ha habido momentos de flaqueza, instantes de incertidumbre y ahogo en los que sólo quería huir (sin saber muy bien porqué o con qué propósito). Pero entonces aparecían ciertas personas maravillosas y el maremágnum de tragedias aparentes se volatilizaba. Y es que una tarde entre amigos puede cambiarlo todo. Gracias a mis pikys de "Fuenla", Carla e Irene, y en especial a Paula (da igual lo lejos que estemos: siempre serás mi camino de regreso). A la Cofradía: Irene, Borja, Buru, Jessi, Pepe, Dani, Pablo Ibarburu, Txarli... sólo diré que me habéis mapacheado el corazón. A los gallegos: Nur, Marta, Fuchu, Cristian y Pablo: sois verano en mi vida. A mi socia María: gracias por estar siempre dispuesta a ayudarme desde el borde de los versos.

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“Se retrocede con seguridad
pero se avanza a tientas.
Uno adelanta manos como un ciego,
ciego imprudente por añadidura,
pero lo absurdo es que no es ciego
y distingue el relámpago la lluvia,
los rostros insepultos la ceniza,
la sonrisa del necio, las afrentas,
un barrunto de pena en el espejo,
la baranda oxidada con sus pájaros,
la opaca incertidumbre de los otros
enfrentada a la propia incertidumbre.
Se avanza a tientas/lentamente
por lo común a contramano
de los convictos y confesos,
en búsqueda tal vez
de amores residuales
que sirvan de consuelo y recompensa
o iluminen un pozo de nostalgias.
Se avanza a tientas/vacilante,
no importan la distancia ni el horario,
ni que el futuro sea una vislumbre
o una pasión deshabitada.
A tientas hasta que una noche
se queda uno sin cómplices ni tacto
y a ciegas otra vez y para siempre
se introduce en un túnel o destino
que no se sabe dónde acaba.”

(Mario Benedetti)

# ÍNDICE

# GLOSARIO DE ABREVIATURAS

| | |
|---|---|
| ABPV | Virus de la parálisis aguda |
| ADN (DNA) | Ácido desoxirribonucleico |
| ADNc | ADN complementario |
| AGID | Inmunodifusión en gel de agarosa |
| AIV | Virus iridiscente |
| ALPV | Virus de la parálisis letal de los ápidos |
| AmFV | Virus filamentoso |
| ARN (RNA) | Ácido ribonucleico |
| BQCV | Virus de la realera negra |
| BVX | Virus X de las abejas |
| BVY | Virus Y de las abejas |
| CBPV | Virus de la parálisis crónica |
| CCD | Colony collapse disorder |
| CGE | Copias de genoma equivalente |
| Ct | Ciclo umbral |
| DWV | Virus de las alas deformadas |
| ELISA | Ensayo de inmunoabsorción ligado a enzimas |
| EV | Virus Egipcio |
| IAPV | Virus de la parálisis aguda israelí |
| IRES | Sitio de entrada para el ribosoma interno |
| KBV | Virus Kashmir |
| LSV-1 | Virus del lago Sinaí-1 |
| LSV-2 | Virus del lago Sinaí-2 |
| LSV-3 | Virus del lago Sinaí-3 |
| LSV-4 | Virus del lago Sinaí-4 |
| LSV-5 | Virus del lago Sinaí-5 |

| | |
|---|---|
| MAPA | Ministerio de Agricultura, Pesca y Alimentación |
| NF-Kb | Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas |
| ORF | Marco de lectura abierto |
| PAGE | Separación por electroforesis en geles de poliacrilamida |
| PCR | Reacción en cadena de la polimerasa |
| qPCR | Reacción en cadena de la polimerasa cuantitativa o en tiempo real |
| RdRp | Polimerasa ARN dependiente de ARN |
| RNAi/iRNA | ARN interferente |
| RNI | Radicales intermediarios de nitrógeno |
| ROI | Radicales intermediarios de oxígeno |
| RT | Retrotranscripción |
| RT-qPCR | Reacción en cadena de la polimerasa con transcriptasa inversa |
| SBPV | Virus de la parálisis lenta de la abeja |
| SBV | Virus de la cría ensacada o cría sacciforme |
| SDC (CCD) | Síndrome de despoblamiento de las colmenas (Colony collapse disorder) |
| SPIA | Single primer isothermal amplification |
| ssRNA | ARN de cadena simple |
| TSBV | Virus de la cría ensacada tailandés |
| TuRSV | Virus del mosaico del nabo |
| VDV-1 | Virus *Varroa destructor*-1 |
| VP | Proteína vírica |
| WB | Western Blot |

# RESUMEN / ABSTRACT

# Resumen

Desde la segunda mitad del siglo XX, se ha podido observar una disminución constante y progresiva en el número de abejas. Estos insectos cumplen múltiples y valiosas funciones, entre las que destaca su labor como polinizadores o el mantenimiento de la biodiversidad de todo el planeta. Además de las repercusiones negativas sobre el sector apícola, las consecuencias de estas pérdidas tienen un alcance mundial, en tanto que la conservación de los polinizadores constituye un factor clave para la agricultura, la producción alimentaria, la sostenibilidad medio ambiental y los ecosistemas.

Existen numerosos factores involucrados en estas pérdidas, entre los que destacan los contaminantes ambientales, los patógenos, los agentes fitosanitarios, las especies invasoras o el cambio climático. Aunque la importancia relativa de cada uno de estos factores continúa siendo una incógnita, en los últimos años se ha demostrado que los patógenos, así como las condiciones medio ambientales y el sistema inmunitario de las abejas, son elementos determinantes para las colmenas. Dentro de los patógenos implicados en estas pérdidas, destacan el ácaro *Varroa destructor* y el virus de las alas deformadas (DWV). Cuando se produce una infección por DWV en colmenas parasitadas por varroa, la respuesta del sistema inmunitario será decisiva. En este contexto, existen otros factores que rodean a la colmena y que serán determinantes para la salud de la misma, como son el medio ambiente y la nutrición. Por ello, la presente tesis doctoral, titulada **"Análisis epidemiológico y molecular de los principales patógenos de *Apis mellifera* y su importancia en el desencadenamiento del colapso de las colmenas"** se ha centrado en el estudio de los principales agentes infecciosos y parasitarios de las abejas en relación con las condiciones medio ambientales y el sistema inmunitario de la colmena. El trabajo desarrollado ha generado cuatro artículos científicos, tres de ellos publicados en revistas científicas indexadas y otro en fase de revisión.

El **primer objetivo** de esta tesis doctoral fue determinar si las diferencias genéticas en la secuencia de DWV podían conllevar modificaciones en la virulencia del virus. Como resultado de este objetivo, se ha proporcionado por primera vez la secuencia completa de dos variantes del virus (DWV-A y DWV-B) en España. Además, se ha evidenciado que la presencia de una u otra variante podría ser determinante para la colmena, en tanto que DWV-A se encontró en mayor medida en aquellas colmenas con peor estado sanitario, mientras que DWV-B fue predominante en colmenas sanas.

El **segundo objetivo** se centró en el estudio del sistema inmunitario de las abejas en relación con el binomio DWV-varroa y la mortalidad de las colmenas. Se seleccionaron cuatro genes representativos de las distintas vías inmunitarias, que fueron analizados junto con parámetros sanitarios. Este trabajo permitió el desarrollo de potenciales marcadores de sistema inmunitario que podrían aportar valiosa información acerca del estado de salud de las colmenas.

El **tercer objetivo** consistió en el estudio de la nutrición y el medio ambiente en relación con la salud de las colmenas. En primer lugar, se evaluó la influencia de la diversidad de polen recolectado por las abejas y sus implicaciones en la salud de las mismas (**sub objetivo 3.1**). Los resultados fueron inesperados, ya que no se observó ninguna correlación entre los marcadores de salud y la diversidad de polen. En segundo lugar, se analizaron las características medio ambientales de los colmenares en base a factores descritos en la bibliografía (**sub objetivo 3.2**). Los colmenares localizados en emplazamientos con peores condiciones para la producción apícola (paisajes fragmentados, presencia de cultivos, etc.) mostraron niveles de patógenos más elevados y peor estado sanitario.

El **cuarto objetivo** se centró en el estudio de DWV y varroa en relación con la fortaleza de las colmenas a lo largo del tiempo. Los resultados obtenidos demostraron que las colmenas con altos niveles de DWV y varroa presentaban menor vigor y menor capacidad de supervivencia. Por lo tanto, se validaron

resultados previos y se enalteció la importancia de la evaluación de estos dos patógenos.

Esta tesis ha supuesto un gran avance en el estudio de algunos de los factores más influyentes en la salud de las colmenas, utilizando para ello una perspectiva multidisciplinar, combinando enfoque molecular, medio ambiental y epidemiológico. Así pues, los resultados obtenidos han aportado nuevas herramientas para el monitoreo y control de los colmenares, profundizando en el conocimiento de aspectos fundamentales como el sistema inmunitario de las abejas o la genética de DWV.

# Abstract

The number of honey bees has been in constant and progressive decline since the second half of the twentieth century. These insects play a key role as pollinators and in the maintenance of global biodiversity. Colony losses include two kind of phenomena. The first one is "Colony collapse disorder" (CCD), which consists of a rapid decrease in bee population, but honey/breeding/pollen stores remain adequate and bees do not show disease symptoms. The second one is winter mortality after wintering. In addition to the impact on the beekeeping sector, colony losses also affect food production, environmental sustainability and ecosystems.

There are many factors involved in CCD and winter losses, such as environmental pollutants, the global expansion of pathogens, phytosanitary agents, invasive species, inadequate management, and climate, among others. Although the relative importance of each of these factors remains unknown, there is an agreement on the importance of pathogens, environmental conditions and bee´s immune system. Among pathogens, the *Varroa destructor* mite and the deformed wing virus (DWV) seem to play a key role in triggering colony losses. When both agents affect the colony, the immune system response is decisive. Other important factors are the environment around the colony and honey bee´s nutrition. Therefore, the present doctoral thesis, entitled "Epidemiological and molecular analysis of the main pathogens of the *Apis mellifera* and their importance in the triggering colony collapse", has focused on the study of the main infectious and parasitic agents affecting honey bee colonies, taking into account the honey bee´s immune system and environmental conditions. The results of the findings of this doctoral thesis have been reflected in four scientific articles, three of them published and the other one under review in scientific journals.

The **first objective** focused on the study of DWV sequence. Here, we have provided the whole sequence for two DWV variants in Spain (DWV-A and

DWV-B). In addition, our results revealed that nucleotide differences may have an impact on virus virulence, since DWV-A levels were higher in colonies with worse health status.

The **second objective** was to study honey bee´s immune system in relation to DWV-varroa and colony mortality. Four immune genes were analysed and compared to health parameters. Potential immune markers were developed, which may provide useful information about colony health.

The **third objective** consisted of the study of honey bee´s nutrition and environment, and their relationship with colony health. First, pollen diversity role in colony health was examined (sub objective 3.1), with no significant results. Second, environmental conditions around the apiaries were evaluated (sub objective 3.2). Worse environmental conditions were related to worse health status.

The **forth objective** focused on the relationship between DWV and varroa with colony strengh over time. Our findings showed that high DWV and varroa levels were associated with poor colony vigour and lower survival capacity. Therefore, previous results from this thesis were validated and the need of evaluating both pathogens was emphasised.

Thus, this thesis represents a major progress in the study of some of the most important factors for colony health from a multidisciplinary perspective, combining molecular, environmental and epidemiological approaches. Therefore, the findings from this thesis have provided new tools for colony monitoring and control, improving the knowledge of key factors like honey bee´s immune system and DWV genetic.

# INTRODUCCIÓN

# 1. Las abejas

## 1.1 Especies de abejas y su distribución

La abeja melífera o abeja de la miel es un insecto perteneciente a la familia *Apidae,* la cual se engloba dentro del orden Hymenoptera. Esta familia abarca alrededor de 40000 especies de abejas, entre las que destacan las del género *Apis*, que comprende nueve especies de abejas sociales. Dentro de este género, las distintas especies se clasifican en tres linajes en función del tipo de cría y el tamaño de las mismas (Figura 1):

- Abejas que construyen sus nidos estableciendo panales paralelos en los huecos de los árboles y otros espacios naturales, lo que facilita al ser humano imitar con facilidad sus panales y, por ello, son las más utilizadas en apicultura. Este grupo engloba cinco especies de abejas: *Apis mellifera, Apis cerana, Apis koschevnikovi, Apis nigrocincta* (abeja melífera de Filipinas) y *Apis nuluensis* (abeja melífera de Borneo).
- Abejas que utilizan espacios abiertos para la construcción del nido, tales como ramas de plantas (como es el caso de las especies *Apis dorsata, Apis florea* y *Apis andreniformis*) o acantilados y estructuras rocosas (*Apis laboriosa*).

Las principales especies utilizadas para apicultura son la abeja asiática *Apis cerana* y *Apis mellifera*. Ambas descienden de un antepasado común, aunque han evolucionado por separado hasta dar lugar a dos especies genéticamente distintas. Se distinguen principalmente por su tamaño, siendo *Apis mellifera* más grande, y por la susceptibilidad a enfermedades, siendo de especial relevancia en el caso de la parasitosis por *Varroa destructor*. Este ácaro, originario de la abeja asiática, fue introducido en Europa en 1985, causando una importante epidemia en las abejas *Apis mellifera*, las cuales no se encontraban adaptadas al mismo.

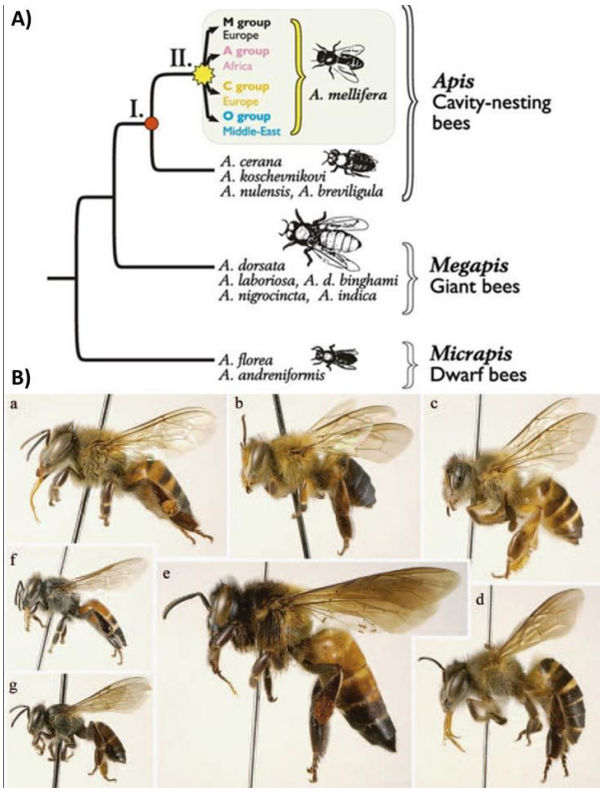


**Figura 1.** Tipos de abeja del género Apis. A) Evolución de los diferentes tipos de abeja: abejas que construyen nidos en oquedades (género *Apis*), abejas gigantes (*megapis*) y abejas enanas (*micrapis*). B) Comparativa de morfología y tamaño de cada una de las especies del género apis. a) *Apis* (*Apis) mellifera*; b) *A.* (*A.*) *koschevnikovi*; c) A. (A.) *nigrocincta*; d) *A.* (*A.*) *cerana*; e) *A.* (*Megapis*) *dorsata*; f) *A.* (*Micrapis*) *florea*; g) *A.* (*M.*) *andreniformis*. Fuente: Engels et al., 2009.

La abeja europea, *Apis mellifera*, actualmente se encuentra distribuida en Europa y África, noroeste de Asia, Oriente Próximo, Cáucaso y la meseta iraní (Figura 2). Debido a las exportaciones de colmenas, su presencia se ha extendido también por América y Australia. Son numerosas las subespecies derivadas de esta especie, siendo las más conocidas *Apis mellifera mellifera* (principal especie de la península ibérica), *Apis mellifera lingüstica* (originaria en Italia y de carácter muy dócil), *Apis mellifera*

*cárnica* (originaria en los Alpes y muy apreciada por su docilidad) y *Apis mellifera caucásica* (originaria en el Cáucaso).

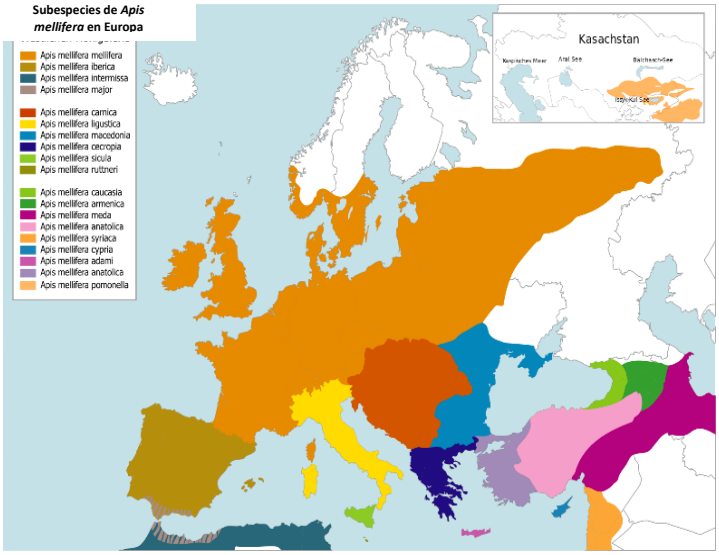


**Figura 2.** Distribución de las subespecies de *Apis mellifera* en Europa. Fuente: Karl Udo Gerth, wikipedia.com, 2014.

## 1.2 La colmena: biología de un súper organismo

Las abejas cuentan con una de las estructuras sociales más particulares del reino animal, lo que condiciona su biología y, con ello, la apicultura como ganadería. En primer lugar, debemos concebir la colmena como un súper organismo y entender que las abejas son piezas de un engranaje mucho mayor. Dentro de la colmena, cada individuo se encuentra altamente especializado y cumple una función que tiene como único fin el porvenir de la propia colonia. La colmena es, por tanto, el conjunto de abejas que derivan de una misma reina, donde se distinguen distintos tipos de castas:

- Abeja reina: se trata de la única hembra fértil de la colmena, siendo la encargada de la reproducción de la colmena. Las reinas se desarrollan en unas celdillas especiales denominadas "realeras" (Figura 3), más grandes y con forma de bellota, y son alimentadas por las obreras con jalea real. Esta

alimentación propicia la fertilidad de la abeja y su mayor tamaño. Tan solo sobrevive una abeja reina por colmena, que saldrá de su celda cuando el clima sea cálido para ser fecundada por los zánganos, tras lo que se dedicará a poner huevos. Gracias a la espermateca que posee, podrá controlar la fecundación de cada huevo, de tal modo que si el huevo está fecundado nacerá una abeja obrera, mientras que si no lo está nacerá un zángano. Para mantener la cohesión de la colmena, la abeja reina emite una serie de feromonas que permiten controlar la producción de más reinas. La esperanza de vida media de una abeja reina es de dos o tres años, aunque pueden llegar a vivir hasta cinco años.

**Figura 3.** Realera (celda de la reina), de forma abellotada y más grande. Bajo ella se distinguen las celdas de las obreras, redondeadas y más pequeñas. Fuente: Rober VS, Pinterest.

- Abeja obrera: encargadas de la mayor parte de las tareas dentro de la colmena, las obreras son la casta más numerosa (Hillyer, 2016; Jean-Prost, 1987). Su ciclo se extiende hasta 21 días: nacen de un huevo y a los tres días se convierten en larvas que operculan la celdilla, pasando a prepupa y pupa, hasta que eclosiona y abre la celdilla (Figura 4). Desde su nacimiento, la abeja obrera pasa por distintas etapas en las que llevará a cabo determinadas

funciones. Primero, las cereras crean y mantienen las celdillas; después se convierte en alimentadoras de la abeja reina y las larvas; posteriormente, se transforma en guardiana y se encarga de proteger la colmena de depredadores y evita la entrada de abejas ajenas; otra función de la abeja adulta es la de ventilar, generando corrientes de aire que mantienen estable la temperatura; finalmente, pasa a ser pecoreadora, momento en el que sale de la colmena y recolecta néctar, polen y agua. Una abeja obrera puede llegar a volar hasta tres kilómetros de distancia. Cuando encuentra una zona de pecoreo adecuada, regresa a la colmena realizando una danza ("danza de las abejas" que tiene por objetivo comunicar al resto de la colmena la posición exacta de la zona con recursos). La vida de las obreras varía en función de la época de nacimiento: las nacidas en enero-febrero viven alrededor de tres meses, las nacidas en abril-mayo 28-40 días, en junio-agosto 80 días, 90 las nacidas en octubre y hasta 140 días las nacidas en noviembre. La mayor esperanza de vida que se observa en las abejas de invierno se debe a que, durante esta época, la puesta de la reina decae estrepitosamente, por lo que se debe garantizar la supervivencia de la colmena mediante la denominada "hibernación". Un estado de salud adecuado para enfrentar esta fase es clave de cara a la supervivencia de la colmena.

- Zánganos: la principal función del zángano es la de fecundar a la reina, aunque se ha postulado que podrían ayudar al mantenimiento de la temperatura de la colmena y repartir el néctar. Se distinguen visualmente dado que tienen mayor dimensión que las obreras, un abdomen cuadrado y los ojos grandes y contiguos (Figura 5).

**Figura 4.** Abeja emergiendo de su celdilla. Fuente: Anthony Bannister, San Francisco Chronicle.


**Figura 5.** Zángano (Z) junto a una abeja obrera (O). Fuente: Juan Simón Padrón, Creative commons.

De manera generalizada, la abeja reina realiza la puesta del huevo, que eclosiona cuatro días después dando lugar a la larva. Dicha larva es alimentada por las abejas nodrizas, aumentando su tamaño hasta que al octavo día se dispone de manera vertical dentro de la celdilla, de tal modo que el extremo anterior queda orientado hacia fuera. Al noveno día, las nodrizas operculan la celdilla y se produce la

metamorfosis. La pupa se transforma en adulta y el día veintiuno emerge de la celdilla. En el caso de los zánganos, el ciclo se extiende a veinticuatro días, mientras que la reina tiene un ciclo más corto, de dieciséis días (Figura 6).

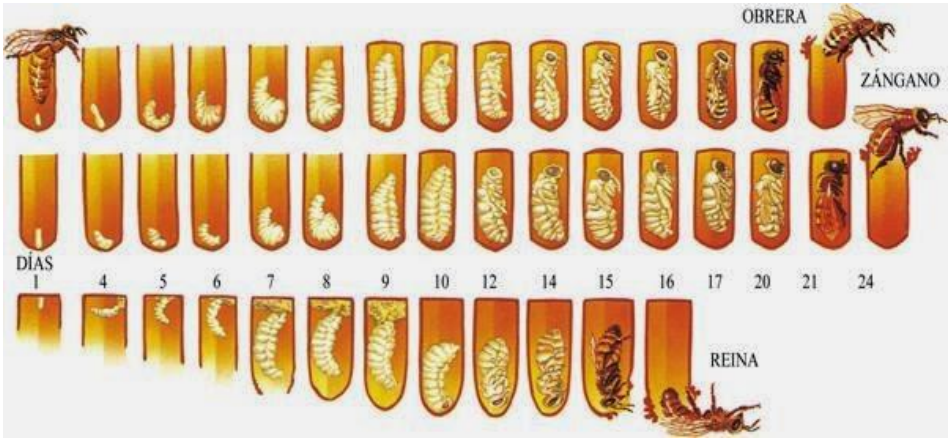


**Figura 6.** Esquema del desarrollo de la abeja obrera, zángano y reina en el interior de la celdilla de cría. Fuente: lareinademicolmena.blogspot.com.

# 2. La apicultura

## 2.1 Breve historia de la apicultura

La apicultura es una de las ganaderías más antiguas que existen, manteniéndose gran parte de sus características fieles a sus orígenes. Las abejas estaban ahí, contribuyendo a la formación de un mundo sostenible mucho antes que nosotros. El análisis etimológico de la palabra "apicultura" nos remite al latín *Apis* (abeja) y *Cultura* (cultivo), y que es definida como el conjunto de técnicas y conocimientos relativos a la cría de las abejas (RAE, 2019).

Al principio, el hombre recolectaba los paneles silvestres en la primavera y los colocaba sobre paneles creados con paja. A finales del verano, tras sacrificar una gran parte de las abejas, se recortaban los panales para obtener miel y cera. Pero no fue hasta los años 8000 y 4000 A.C. cuando empezó a desarrollarse la apicultura,

encaminándose hacia la práctica apícola que hoy en día conocemos. Se modificó la pauta de recolección de las colmenas y se empezó a aportar un habitáculo a las abejas fabricado con distintos materiales, para que éstas lo anidasen y poder extraer sus productos.

Las primeras evidencias del contacto del ser humano con las abejas se encuentran en el mesolítico, donde pinturas rupestres mostraban la captura de paneles silvestres. Los jeroglíficos egipcios también hacen acopio de las prácticas apícolas, quienes solían decir que, cuando el Dios del Sol lloraba, sus lágrimas se convertían en abejas al rozar el suelo. Posteriormente, los fenicios, griegos, romanos y árabes continuaron descubriendo el apasionante mundo de la apicultura, quedando constancia de la primera evidencia escrita de la apicultura en España en el año 1100 A.C, en el Imperio Tarteso, asentado en Andalucía. En la Edad Media, Alfonso X realizó las primeras ordenanzas sobre apicultura y, posteriormente, en el siglo XIV, Méndez de Torres estudia la reproducción de las abejas.

La apicultura moderna surge a partir del siglo XVIII, gracias a los avances científicos que permitieron conocer la biología y el comportamiento de las abejas a nivel individual, y de la colonia a nivel de súper organismo. Desde que Nikel Jacob descubre en 1568 que las abejas crían reinas a partir de huevos de larvas jóvenes, pasando por la invención de la colmena tipo Langstroth en 1851 y terminando en la actualidad, muchas innovaciones han sido llevadas a cabo en el sector apícola. Sin embargo, por tratarse de una ganadería muy especial, fuertemente ligada al ciclo natural de las abejas, existen una serie de características que se conservan.
Actualmente, distinguimos dos tipos de apicultura:

- Apicultura sedentaria o estante**:** el asentamiento apícola se mantiene en la misma ubicación durante todo el ciclo, aportándose alimentación artificial en determinadas épocas del año.

- Apicultura trashumante**:** el colmenar se traslada a otra ubicación, buscando así las zonas más apropiadas, con floración propicia para las abejas. De este modo, se garantiza el aprovechamiento máximo de los recursos disponibles.

## 2.2 El sector apícola en España

Si nos centramos en España, la producción de miel se sitúa en torno a 31.018 Tm al año (MAPA, 2018). En 2012 se produjo un acusado descenso en producción probablemente debido a las malas condiciones climáticas, lo que se repitió en 2016. Los resultados obtenidos en las campañas del 2017 y 2018 fueron similares a los de los años anteriores, dado el bajo rendimiento de los colmenares, asociado, de nuevo, a un mal clima. Sin embargo, España sigue siendo uno de los principales productores de miel de la Unión Europea y el país con un mayor número de apicultores profesionales (definidos como aquellos cuyas explotaciones cuentan con más de 150 colmenas). La producción de cera, sin embargo, se mantiene estable desde hace dos décadas.

En cuanto a la distribución de la producción en el territorio nacional, ésta se concentra fundamentalmente en 4 CCAA: Andalucía (20%), C. Valenciana (12%), C. León (16%) y Extremadura (22%) (Figura 7). Entre las 4 concentran más del 60% del total de la producción de miel en España (MAPA, 2018). No obstante, el principal valor de esta actividad agraria radica en su importante contribución al mantenimiento de los ecosistemas y la biodiversidad a través de la polinización entomófila realizada por la cabaña apícola.

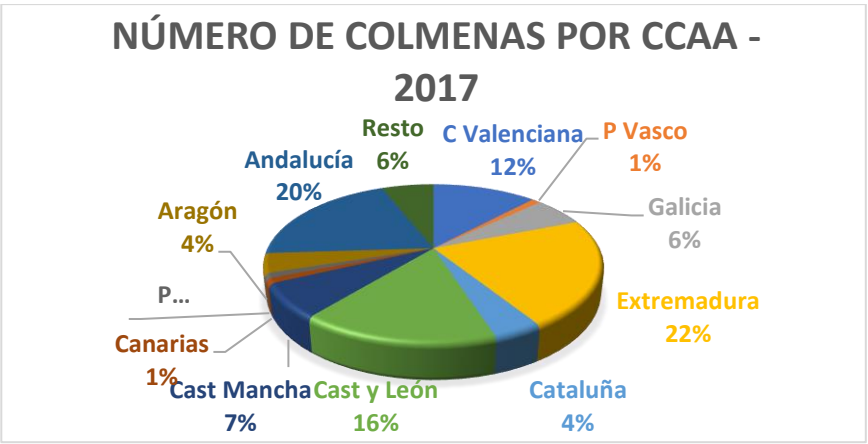


**Figura 7.** Número de colmenas por Comunidad Autónoma. Fuente: elaboración propia a partir de MAPA, 2018.

## 2.3 Censos y explotaciones

El número de apicultores en España es de 31.527, según los últimos datos registrados, de los cuales más de un 18% son profesionales. A pesar de que el número de explotaciones apícolas ha aumentado en un 40% en los últimos años para intentar paliar las pérdidas acusadas de los años anteriores (Figura 8). Sin embargo, con la intensificación de los cultivos, las necesidades de polinización también se han visto incrementadas, por lo que el problema no ha sido solucionado.

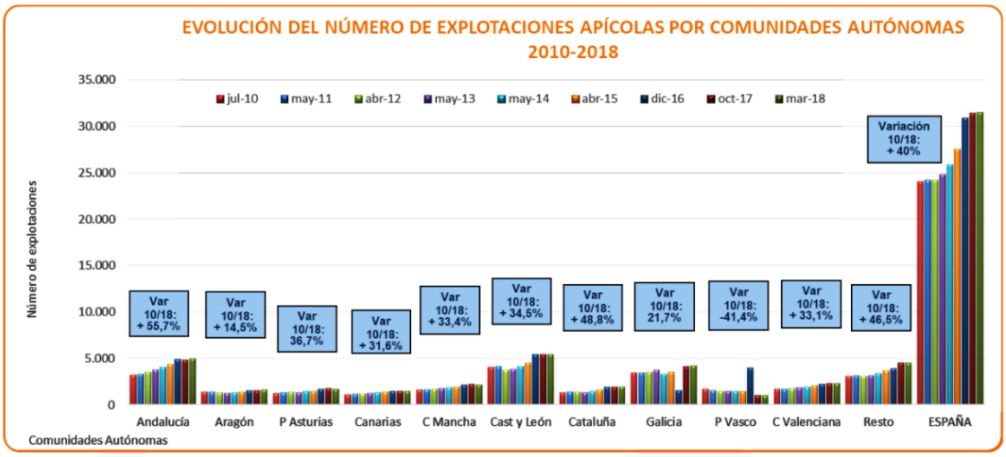


**Figura 8.** Evolución del número de explotaciones apícolas por CCAA. Fuente: MAPA, 2018.

Existe, sin embargo, una gran heterogeneidad dentro del sector: mientras que en la zona norte y noroeste encontramos una apicultura de tipo "hobbista" (apicultores pequeños que, en su mayoría, no practican la trashumancia), en la zona del centro y sur/sureste de la península se practica una apicultura con alto grado de profesionalización y mayoritariamente trashumante.

# 3. Importancia de las abejas

## 3.1 Polinización

A pesar de todos los beneficios que se obtienen a partir de los productos derivados de la colmena, la principal y más importante labor que cumplen las abejas es la polinización (Figura 9). Entendemos por polinización la transferencia de los granos de polen (las células sexuales masculinas de una flor) desde la antera a la superficie receptiva o estigma (órgano femenino de la flor). Aunque algunas especies de plantas se autofertilizan o dependen del viento, la mayor parte requieren de la intervención de insectos polinizadores. Las abejas son la principal especie encargada de realizar esta importante tarea, habiéndose especializado hasta el punto de que una sola colmena es capaz de fertilizar millones de flores en un solo día (Klein et al., 2007). Su eficiencia se debe a diversas razones: sus cuerpos presentan pelos a los que se adhiere el polen, permitiendo su transporte; visitan gran variedad de plantas y con una elevada frecuencia para conseguir el néctar con el que alimentar a las larvas; su tamaño les permite acceder a distintos tipos de plantas; finalmente, su manejo resulta relativamente fácil, pudiendo incrementar el ser humano el número de colmenas y aprovecharse así de su labor. Muchos de los cultivos destinados a la alimentación humana o de animales de producción son polinizados gracias al movimiento específico de las colmenas a lo largo de la temporada apícola (trashumancia).

La apicultura asociada a la polinización es una práctica que requiere una serie de requisitos por parte del apicultor. Se deben seleccionar colmenas fuertes y resistentes, que produzcan una cifra suficiente de abejas obreras de tamaño acorde a

los cultivos que se pretenden polinizar. En Estados Unidos, un gran número de cultivos dependen directamente de la apicultura para ser polinizados, como, por ejemplo, la almendra en California. En España, sin embargo, un porcentaje muy bajo de los apicultores dedican sus colmenas exclusivamente a esta función.

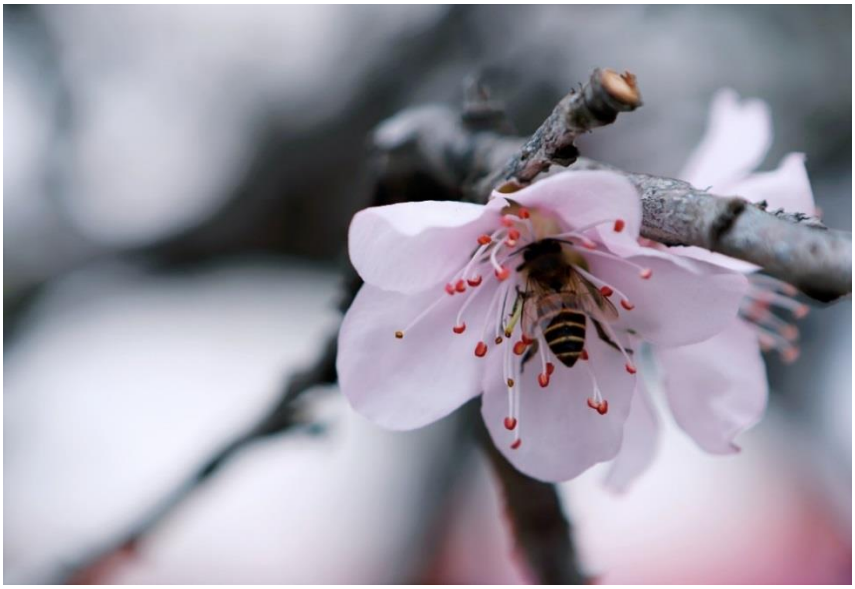

**Figura 9.** Abeja visitando una flor para recolectar polen y néctar (Fuente: pexels.com)

Algunos de los cultivos en los que se emplean las abejas como polinizadoras son la alfalfa, manzana, mora, arándano, tréboles, pepino, melón, nectarino, girasol, calabacín y calabaza, entre otros. Otros cultivos, a pesar de no depender directamente de estos insectos para su crecimiento, se ven igualmente beneficiados por la acción de las abejas. Se estima que aproximadamente 3 millones de ventas anuales de productos agrícolas en todo el mundo dependen de las abejas, lo que se correspondería con un 35% de las calorías consumidas por el ser humano cada año. Además, contribuyen notablemente a la alimentación del ganado doméstico.

Otro beneficio derivado de la polinización es el mantenimiento de la biodiversidad en el planeta. Mediante su acción polinizadora, las abejas y otros polinizadores favorecen el cruce de especies y permiten la supervivencia de plantas

silvestres y en peligro de extinción. Estos procesos aseguran, entre otras cosas, un suministro constante de agua depurada o de aire respirable. Las abejas contribuyen también a la formación de los suelos y el ciclo de los nutrientes, tan necesarios para la agricultura. Además, la biodiversidad es la base de la mitad de la economía mundial, y las medidas encaminadas a su conservación y restauración se perfilan como algunas de las actividades que más se desarrollarán y que más empleo crearán en el futuro (Potts et al., 2010).

## 3.2 Desaparición de las abejas

Pese a que los datos censales y de producción podrían parecer adecuados, la alta demanda de polinizadores, junto con el incremento de las tasas de mortalidad en las colmenas, han logrado que se detonen las alarmas. La opinión pública se ha hecho eco de ello y, en los últimos años, resuena el eco de una amenaza: las abejas están desapareciendo. A partir de la segunda mitad del siglo XX, el número de colmenas en Estados Unidos y Europa ha experimentado un descenso constante hasta llegar a cifras alarmantes. El 19 de mayo de 2018, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) quiso llamar la atención de la población sobre el riesgo que su decrecimiento representa para la diversidad alimentaria.

Según el informe de resultados del programa de vigilancia sobre la pérdida de abejas en España (MAPA, 2017), la mortalidad invernal en España fue del 10,2% en el periodo comprendido entre 2012-2013, con una importante variación a nivel geográfico. Las mortalidades más elevadas se registraron en el oeste peninsular mientras que las menores en el este. En 2013-2014, se redujo notablemente la mortalidad (5,5%), para ascender de nuevo en el periodo 2014-2015, alcanzando el 11,22% (MAPA, 2017). Los patrones detectados se asemejaron a los de los países del centro y sur europeo, según se determinó en el programa EPILOBEE. También se han documentado descensos poblacionales en enjambres silvestres (Moritz, 2007).

Teniendo en cuenta que dichas pérdidas se han producido en periodos de tiempo muy cortos, incluso en la misma temporada, la gravedad del problema se incrementa. A pesar de que los episodios de pérdidas inusuales de las colmenas han sido documentados a lo largo de la historia de la apicultura, nunca se habían vuelto tan frecuentes como en las dos últimas décadas.

## 3.3 Síndrome de despoblamiento de las colmenas

En los últimos años se ha acuñado el término "síndrome de despoblamiento de las colmenas" (SDC o CCD por sus siglas en inglés). Se trata de un problema de causa multifactorial caracterizado por:

- Disminución rápida del número de abejas obreras adultas, quedando las colmenas en mal estado o despobladas. En éstas, sin embargo, se observa abundante cría en comparación con la población adulta y reservas de miel y polen (Figura 10).
- Suele producirse en la primavera, tras la invernada.
- No se observan abejas muertas ni dentro ni alrededor de la colmena.
- Ausencia de signos clínicos de enfermedades. Tampoco se observan especies invasoras, como el escarabajo de la colmena o avispas, ni pillaje (Cox-Foster et al., 2007; vanEngelsdorp, Evans, et al., 2009).

**Figura 10.** Cuadros de cría con insuficiente cobertura de abejas, indicando la rápida pérdida de abejas adultas. Fuente: vanEngelsdorp, 2009. (Colony Collapse Disorder: A Descriptive Study).

Desde mediados del siglo XX, asociado al comienzo del uso masivo de los pesticidas, comenzaron a notificarse pérdidas inusuales de colmenas de abejas en Estados Unidos y, posteriormente, en Europa (Figura 11). Sin embargo, no fue hasta 2006 cuando se acuñó el término SDC y se prestó mayor atención al problema, investigando sus posibles causas. Desde entonces, las pérdidas de colmenas han continuado creciendo hasta el punto de tratarse de una "crisis mundial" (vanEngelsdorp et al., 2009; vanEngelsdorp, Hayes, Underwood, & Pettis, 2009; vanEngelsdorp & Meixner, 2010). Sin embargo, en los últimos años ha descendido el número de casos registrados en Estados Unidos, probablemente asociado a la labor de los apicultores que, mediante la división de sus colonias, han logrado mantener el número de colmenas estable.

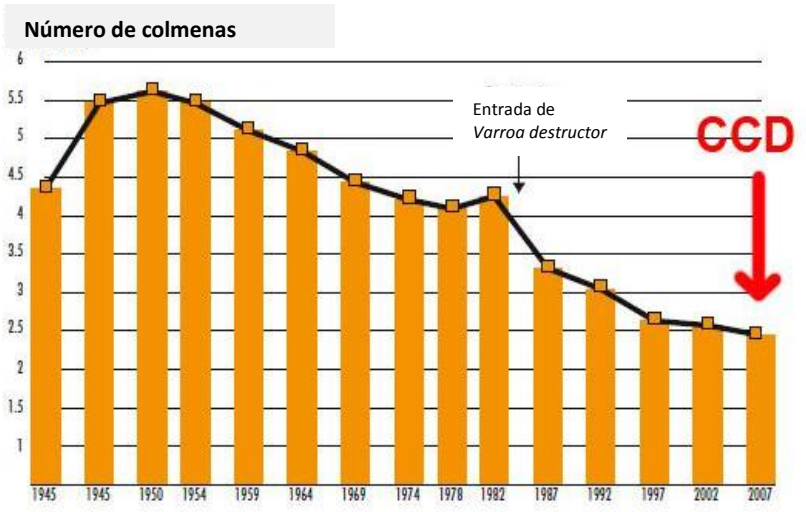


**Figura 11.** Evolución del número de colmenas en Estados Unidos. Como se observa en la gráfica, la entrada del parásito *V. destructor* supuso un gran descenso en el número de las mismas. Fuente: US Deparment of Agriculture (USDA) National Agricultura Statistics Service (NASS) NB.

## 3.4 Pérdidas invernales

Una gran parte de las pérdidas en las colmenas se observan durante el invierno, por lo que recientemente se utiliza el término "mortalidad invernal" para referirse a las muertes que suceden durante dicho periodo o tras la invernada. De acuerdo con el programa EPILOBEE (Antoine Jacques et al., 2017) llevado a cabo en Europa, los rangos de mortalidad invernal varían entre países miembros, situándose entre el 2.4 y el 15.4% en el año 2014 (Figura 12). Al igual que el SDC, se trata de un problema de carácter multifactorial y de sintomatología inespecífica. La mortalidad

invernal en España fue del 9,8% en el periodo 2016-2018, muy similar a los años previos, sin apreciarse variación clara por territorios.

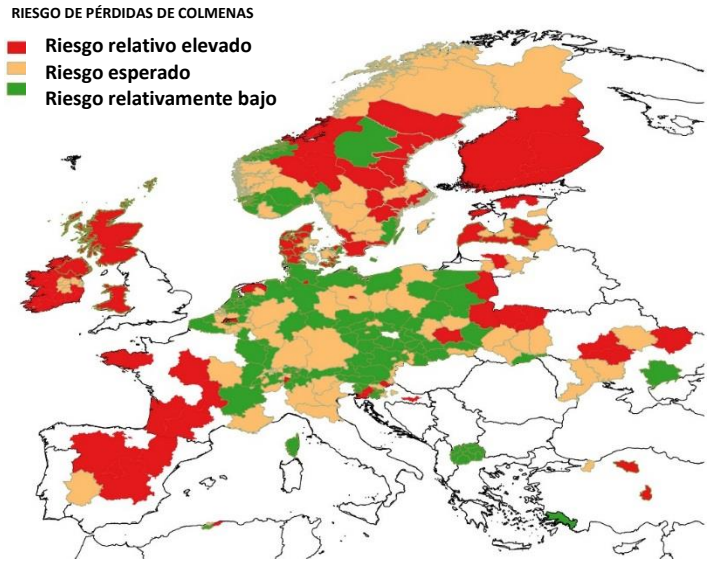


**Figura 12.** Riesgo relativo de pérdidas invernales según país en 2015-2016 en Europa. Fuente: EPILOBEE Project, 2016 (Preliminary analysis of loss rates of honey bee colonies during winter 2015/16 from the COLOSS survey).

# 4. Factores implicados en las pérdidas de las colmenas

A la hora de estudiar las pérdidas de las colmenas, se debe tener en cuenta que se trata de un problema de carácter multifactorial, lo que dificulta la puesta en marcha de medidas que permitan controlar las causas involucradas. Se debe aplicar, por tanto, un enfoque global que tenga en cuenta las distintas variables implicadas.

Las principales causas de las pérdidas en las colmenas (SCD y mortalidad invernal) son las siguientes:

## 4.1 Factores medio ambientales

La colmena es un súper organismo totalmente dependiente del medio ambiente que la rodea (temperatura, disponibilidad de floración, presencia de masas de agua, precipitaciones...), lo que condiciona de manera determinante la producción apícola. La falta de biodiversidad y el clima, por ejemplo, cumplen un importante papel a la hora de propiciar la presencia de alimento. A su vez, la pérdida, fragmentación y deterioro del hábitat conlleva una disminución de los recursos disponibles para las abejas, lo que disminuye la calidad y disponibilidad de polen, induciendo como consecuencia estrés nutricional. Por ejemplo, las grandes extensiones de monocultivos contribuyen a que las abejas sólo tengan alimento en periodos del año muy restringidos. Otros factores desencadenantes de estrés en las colmenas son la intensificación de la agricultura, los cambios en la cobertura vegetal o la falta de néctar y polen.

Por otro lado, las condiciones climáticas tienen un importante efecto en la viabilidad y productividad de las abejas, tanto a nivel individual como del conjunto. Se debe garantizar una temperatura constante en el interior de la colmena, sobre todo en el centro, donde se sitúa la cría. De este modo, climas fríos disminuyen la actividad de la colmena, mientras que el calor favorece el crecimiento de la cría y la actividad de pecoreo. Además, el clima va a determinar el entorno que rodea a la colmena, pudiendo producir un deterioro del mismo.

Todos estos factores medioambientales cumplen una importante labor a la hora de mantener o alterar el equilibrio entre los patógenos y el hospedador, de tal modo que pueden propiciar el paso de una infección encubierta a una infección aparente con desarrollo de síntomas.

## 4.2 Pesticidas

Los avances y la intensificación en la agricultura han propiciado el uso masivo de pesticidas en los últimos años, llegando a convertirse en una importante amenaza

para las abejas. Éstas, durante la actividad de pecoreo, quedan expuestas a los herbicidas e insecticidas que se utilizan para tratar los cultivos. La cercanía a cultivos intensivos puede ser, por tanto, un factor determinante que favorezca la exposición de las colmenas a este tipo de productos.

En los últimos años, los pesticidas en general y los neocotinoides en particular, han sido apuntados como una de las principales causas implicadas en las crecientes pérdidas de las colmenas. Por ello, muchos estudios se han centrado en dilucidar el papel de los mismos en el síndrome de despoblamiento. El efecto agudo de este tipo de productos puede ocasionar la muerte de la colmena con relativa facilidad, aunque es poco frecuente que se produzca una exposición de este tipo. Mucho más frecuente es, sin embargo, la exposición crónica, que puede tener efectos sub-letales, entre los que se encuentran la parálisis, desorientación y cambios de comportamiento (Desneux, Decourtye, & Delpuech, 2007). Como consecuencia de este efecto subletal, el sistema inmunitario de las abejas podría verse comprometido, por lo que éstas serían más proclives a sufrir otro tipo de problemas sanitarios. No obstante, existe una gran controversia al respecto en la comunidad científica (Sánchez-Bayo et al., 2016).

## 4.3 Nutrición inadecuada

Además del néctar, que se compone casi en su totalidad de carbohidratos, la principal fuente de nutrientes de las abejas es el polen, que proporciona proteínas, aminoácidos, colesterol, lípidos, ácidos esenciales, vitaminas y minerales.

Una adecuada nutrición es clave para la supervivencia y productividad de la colmena. Una alimentación deficiente, especialmente durante el invierno, puede ocasionar graves pérdidas productivas e incluso la muerte de la colmena. Cuando los recursos son escasos y no existen reservas suficientes para soportar la invernada, es muy frecuente encontrar abejas muertas alrededor de la colmena y en el interior de las celdillas. Por ello, resulta fundamental revisar las colmenas durante esta etapa,

con el fin de evaluar si los recursos son suficientes o aportar alimentación suplementaria en el caso de que sea necesario.

El alimento debe ser suficiente en cantidad, pero también en calidad, de tal modo que proporcione a las abejas los nutrientes requeridos. Este hecho es especialmente importante en el caso de los aminoácidos, cuya ingesta debe ser garantizada mediante el consumo de polen. Recientes estudios han apuntado la importancia de determinados nutrientes en el funcionamiento del sistema inmunitario y el desarrollo de procesos fisiológicos (C Alaux, Ducloz, Crauser, & Le Conte, 2010), lo que podría estar relacionado la capacidad para enfrentarse a las enfermedades. Sin embargo, con el incremento de los monocultivos y la fragmentación de los paisajes, la disponibilidad de alimento se ha visto empobrecida en los últimos años, provocando deficiencias nutricionales en las abejas y, con ello, al declive de las mismas. Por ello, resulta necesario examinar la posible interacción entre la nutrición y el desarrollo del sistema inmunitario, lo que podría verse reflejado en la respuesta defensiva frente a enfermedades.

## 4.4 Depredadores y plagas

Dentro de los depredadores y plagas que pueden atacar a las abejas, encontramos los abejarucos, las avispas y los avispones, ratones, arañas, escorpiones, etc. La incidencia de este tipo de amenazas en las colmenas dependerá de las condiciones climáticas y la localización geográfica de las mismas.

- **Roedores**

  Estos pequeños mamíferos pueden ocasionar graves problemas en las colmenas. Se introducen en éstas en el otoño para pasar el invierno, dañando los panales y marcos al construir sus nidos. Para evitar la invasión, se aconseja reducir el tamaño de la piquera con láminas de metal o bloques de madera.

- **Abejaruco (*Merops apiaster*)**

Se trata de un grupo de aves que se alimentan de himenópteros venenosos como las abejas. Estos pájaros atacan a las abejas principalmente en la época de verano, alimentándose de las abejas y pudiendo ingerir a la reina, por lo que es frecuente encontrar colmenas zanganeras (colmenas sin reina ni obreras) tras el ataque del abejaruco. Para prevenir sus ataques, el apicultor debe tener en cuenta la elección de los asentamientos y proteger las colmenas con mallas de sombreo o redes plásticas.

- **Avispas y avispones**

Estos insectos del orden Himenoptera actúan de manera natural como depredadores de las abejas. Hasta el año 2010, la principal avispa en España era la *Vespa crabro,* especie autóctona que no solía provocar graves daños en las colmenas. Sin embargo, desde que se produjo la entrada de la *Vespa velutina,* esta voraz especie invasora ha ocasionado la muerte de multitud de colmenas (Figura 13). Actualmente se encuentra en siete regiones del norte peninsular y en Baleares, donde ha ocasionado grandes estragos. El mecanismo de actuación de la avispa es matar a las abejas con un golpe mandibular y trocearlas para transportarlas hasta su nido, donde servirán de alimento a las larvas. Por su agresividad y capacidad de invasión, en los últimos años se ha generado una intensa alarma entre los apicultores de las regiones afectadas (principalmente el norte peninsular).

## 4.5 Patógenos

Por tratarse de una de las principales causas implicadas en las pérdidas de las colmenas (tanto en el SDC como en la mortalidad invernal), los patógenos serán descritos en el siguiente apartado.

**Figura 13.** Avispa asiática *(Vespa velutina*) acercándose a una abeja. Fuente: Danel Solabarrieta, Creative Commons.

# 5. Patógenos

Son muchos los agentes patógenos que afectan a las abejas. A pesar de la multitud de factores involucrados en las pérdidas de las colmenas, la comunidad científica coincide en que los patógenos (agentes infecciosos o parasitarios que ocasionan enfermedad) son una de las principales causas de mortalidad de las abejas. Numerosas publicaciones científicas han demostrado una mayor presencia y carga de patógenos en colmenas colapsadas (Cepero et al., 2014; Cox-Foster et al., 2007; vanEngelsdorp, Hayes, et al., 2009) lo que incrementa la necesidad de su estudio. Resulta imprescindible, por tanto, elucidar el papel concreto de cada agente en el desarrollo de pérdidas en las colmenas, para tratar de establecer pautas preventivas que ayuden a evitar los daños derivados de su acción. Muchos patógenos se encuentran de manera habitual en las colmenas; sin embargo, ante condiciones de estrés o desequilibrio dentro de la colmena, se puede favorecer el aumento de la virulencia de estos agentes, lo que puede ocasionar graves daños y pérdidas.

## 5.1 Bacterias

Existen varias enfermedades bacterianas descritas en las abejas (Bailey & Ball, 1991), de las cuales destacan las "loques", enfermedades de declaración obligatoria que afectan a la cría y cuyos agentes etiológicos son las bacterias *Paenibacillus larvae* (loque americana) y *Melisococcus plutonius* (loque europea). Ambas enfermedades ocasionan grandes pérdidas económicas y mortandad en las colmenas afectadas, debido a su alta capacidad de contagio (por uso de material apícola de una colmena a otra, comercio de reinas, alta densidad de las colmenas...), siendo la loque americana de mayor virulencia (Figura 14). Estas bacterias provocan la muerte de la cría rápidamente, que se torna de color amarillento y desprende un fuerte olor. Debido a la particularidad de su sintomatología, el diagnóstico de la enfermedad suele realizarse de manera visual. Eliminar estas bacterias de las colmenas es muy complicado, debido a que producen esporas de elevada resistencia, siendo necesario someterlas a temperaturas muy altas para su destrucción.

**Figura 14.** Celdas hundidas con gotas de humedad por loque americana. Fuente: beeinformed.org.

## 5.2 Hongos

La principal enfermedad fúngica que afecta a las abejas es la ascosferosis o cría yesificada. Esta enfermedad, también conocida como "pollo escayolado" por el aspecto que genera en las abejas afectadas, está producida por el hongo *Acosphaera apis.* Se trata de una micosis invasiva que solo afecta a la cría, generando pérdidas significativas tanto en el número de abejas como en la productividad de las mismas (Yakobson, 1991). Las larvas se infectan al ingerir al hongo con el alimento, las esporas germinan en el tracto intestinal y se produce el crecimiento de los micelios de manera intersegmentaria desde el interior de la larva hasta el exterior, dando lugar a una "momia" de color blanquecino al principio. Posteriormente, la larva adquiere un color negruzco debido a la formación de cuerpos fructíferos. Se trata, sin embargo, de una enfermedad oportunista que se presenta cuando las condiciones no son adecuadas, como en épocas de frío, excesiva humedad o alta parasitación por *Varroa destructor.* Esta enfermedad no afecta a las abejas adultas, aunque éstas pueden actuar como fuentes de infección para las larvas al alimentarlas.

## 5.3 Protozoos

Existen una serie de protozoos que pueden ocasionar enfermedades en las abejas adultas, aunque normalmente se encuentran en las colmenas sin producir daño alguno, y sólo bajo determinas condiciones (causas predisponentes) tiene lugar su efecto patógeno.

El protozoo que presenta una mayor incidencia en las colmenas es *Malpighamoeba mellificae,* agente productor de la amebosis. Mediante la ingestión de los quistes, el protozoo llega al intestino, donde tiene lugar la fase de vida móvil, para después dirigirse al sistema excretor. Sin embargo, el desarrollo de sintomatología es poco frecuente.

Otro protozoo que afecta a las abejas es *Chritidia mellificae*, el cual ha sido apuntado como posible predictor de la mortalidad de las colmenas durante el invierno (Ravoet et al., 2013).

## 5.4 Parásitos

Algunas de las enfermedades producidas por parásitos son las siguientes:

- **Acarapisosis**

Se trata de una enfermedad de declaración obligatoria para la OIE, producida por el ácaro *Acarapis woodi* (Figura 15). Este ácaro se aloja en la tráquea de las abejas y se alimenta de su hemolinfa, ocasionando que las abejas dejen de volar e incluso provocando su muerte cuando las infestaciones son elevadas (Bailey & Ball, 1991)

- **Aethinosis**

*Aethina tumida*, llamado el "escarabajo de las colmenas", es una especie africana que invade las colmenas produciendo graves daños en las abejas (Figura 15). Aunque actualmente no se encuentra en España, en Europa se han detectado varios brotes: uno en Portugal en 2004 y, recientemente, en Italia en 2014 y otro en 2016 (OIE, 2019).

- **Tropilaepsosis**

Los ácaros de la especie *Tripilaelaps spp* son parásitos externos de las abejas que se alimentan de su hemolinfa (Figura 15), produciendo malformaciones y hasta el 50% de mortalidad en las larvas (OIE, 2012).

**Figura 15.** Imágenes de *Acarapis woodi* (izquierda), *Aethina tumida* (centro) y *Tropilaelaps clareae* (derecha). Fuente: National Bee Unit, ntnemdept.ufl.edu y USDA.

- **Nosemosis**

La nosemosis es una enfermedad producida por dos especies de microsporidios del género Nosema: *Nosema apis* (patógeno natural de *Apis mellifera*,) y *Nosema cerana* (patógeno natural de *Apis cerana*). A pesar de que existe una especie específica para la abeja europea, recientemente se ha observado una expansión de *Nosema cerana* a territorios europeos, lo que ha producido el declive de *Nosema apis* (Fries, 2010; Paxton, Klee, Korpela, & Fries, 2007). Este microsporidio es un parásito intracelular obligado que invade las células intestinales de las abejas y produce esporas que pueden permanecer inertes durante mucho tiempo (Figura 16). La infección de produce de forma horizontal, mediante ruta fecal-oral, de tal modo que las esporas germinan en el ventrículo, produciendo inflamación y daño en las células epiteliales del intestino. El síntoma más característico es la presencia de heces diarreicas en la piquera (acceso) de las colmenas, aunque es poco frecuente observarlo este síntoma. Más preocupante es su efecto a largo plazo: reducción de la esperanza de vida de las abejas, disminución de la productividad y aumento de la mortalidad en invierno (Fries, Ekbohm, & Villumstad, 1984). Normalmente, las esporas están presentes en colmenas aparentemente sanas y germinan cuando la colmena se encuentra débil. Por todo ello, este microsporidio ha sido señalado como uno de los principales causantes del SDC y de la mortalidad invernal de las colmenas (Higes et al., 2008; Higes et al., 2009; Higes, Martín, & Meana, 2006). Sin embargo, existe controversia al respecto, ya que es frecuente aislar al microscoporidio en colmenas sanas (Chen, Evans, Smith, & Pettis, 2008; Fernandez et al., 2012; Fries, 2010;

Invernizzi et al., 2009; Siede, Berg, & Meixner, 2008). Existen evidencias de la capacidad de este microsporidio para transmitir al virus de las realeras negras (Black Queen Cell Virus, BQCV) (Allen & Ball, 1996; Bailey, Ball, & Perry, 1983).

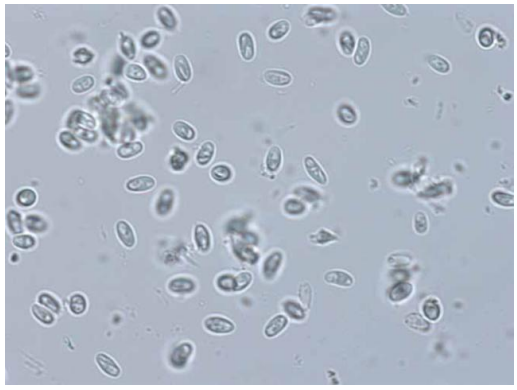

**Figura 16.** Esporas de *Nosema* spp en microscopio. Fuente: food4farmers.org.

- **Varroosis**

La varroosis es una enfermedad producida por el ácaro *Varroa destructor* (Figura 17), parásito originario de la abeja asiática *Apis cerana*, que pasó a ser un parásito de la abeja europea *Apis mellifera* como consecuencia de la coexistencia de ambas especies en el Este de Rusia y en Oriente (Rosenkranz, Aumeier, & Ziegelmann, 2010).

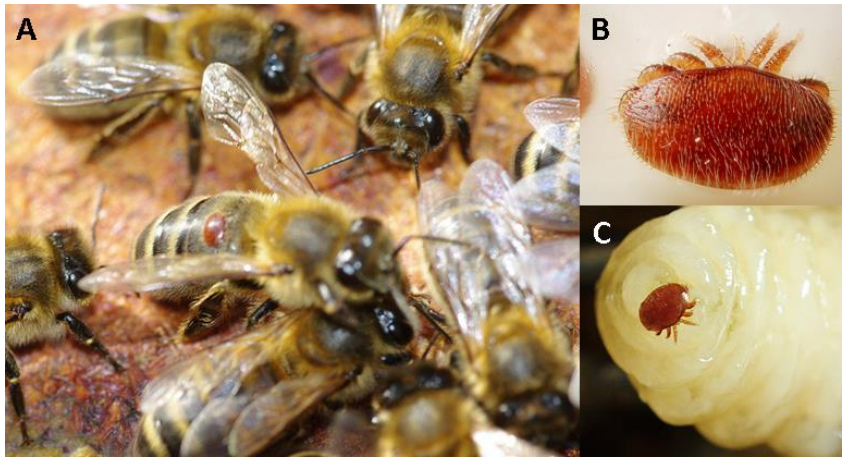


**Figura 17.** Imágenes de *Varroa destructor*. A) Parásito sobre abeja adulta. B) Detalle de hembra de varroa. C) Varroa sobre pupa de abeja.

Las consecuencias de que la varroa diese este salto interespecie han sido devastadoras para las abejas europeas y estadounidenses, convirtiéndose en una de las mayores amenazas a la que la apicultura debe enfrentarse hoy en día (Goulson et al., 2015; Mondet et al., 2014). Se cree que el ácaro llegó a Europa en los años 70, entrando en España en el año 1985 (Pajuelo, 1988). Recientemente, se ha detectado la presencia del parásito en Nueva Zelanda (2000) (Mondet et al., 2014) y en Hawai (2007) (Martin et al., 2012). Actualmente, el único territorio con colmenas de *Apis mellifera* libre de varroa es Australia. Las pérdidas ocasionadas por el parásito han sido catastróficas, llegando a ocasionar la muerte de multitud de enjambres silvestres y hasta un 40% de la cabaña apícola en España (Llorente, 2003). Actualmente, el control de la varroa recae directamente en los apicultores, que deben establecer una serie de pautas e implantar tratamientos antiparasitarios para mantener la población del ácaro bajo control. Para ello, se ha desarrollado una estrategia de control integral basada en la aplicación de tratamientos farmacológicos combinados con un adecuado manejo de las colmenas, con el objetivo de minimizar al máximo los daños ocasionados por el parásito. Tal es la importancia de esta enfermedad que los apicultores están obligados a efectuar al menos un tratamiento al año con los fármacos autorizados por el MAPA (Real Decreto 608/2006). Sin embargo, a pesar de la lucha llevada a cabo contra el ácaro, éste sigue provocando el colapso de un gran número de colmenas todos los años (Martin, 2001; Martin et al., 2012; Schroeder & Martin, 2012; Sumpter & Martin, 2004a).

El número de ácaros aumenta al mismo tiempo que la cría, principalmente al final de la estación. El ciclo de vida del parásito es dependiente de la temperatura y la humedad, pudiendo variar entre días a unos pocos meses. En general, los niveles de infestación más altos se alcanzan al finalizar el verano y durante el otoño, por lo que los tratamientos y las prácticas de manejo en el colmenar deben ir enfocados a evitar un aumento significativo del ácaro en este periodo.

La patología ocasionada por la varroa afecta tanto a la cría como a la abeja adulta, por lo que sus consecuencias son más graves al afectar a ambas etapas fisiológicas. La transmisión directa del ácaro se produce de forma horizontal por contacto entre abejas, por el desplazamiento de las abejas y crías infectadas, así como por productos y material apícola infestado. La transmisión indirecta transovárica se produce de la madre a la cría (Amiri et al., 2018). Los daños derivados de su patogenia son:

- Daño directo: la varroa se alimenta de la hemolinfa de las abejas, principalmente de las larvas y pupas, produciendo la muerte de las mismas o un descenso de la esperanza de vida cuando las abejas alcanzan la madurez. Algunos síntomas característicos de las larvas que han sobrevivido a la infestación son acortamiento de abdomen o alas deformadas. Además, se ha descrito un posible efecto inmunosupresor de la varroa a nivel individual (Yang & Cox-Foster, 2005). Sin embargo, existe controversia al respecto, ya que las publicaciones más recientes sugieren que dicho efecto inmunosupresor puede ser debido más a su relación con el virus de las alas deformadas (deformed wing virus, DWV) que al propio ácaro (Knight, 2014).

- Daño indirecto: además de su capacidad para producir daño en las abejas al alimentarse de su hemolinfa, es conocido el papel de la varroa como transmisor de varios virus de las abejas. Algunos de los virus que son transmitidos por el ácaro son el virus de la cría ensacada (sadcroob bee virus, SBV), el complejo de los virus de la parálisis formado por el virus de la parálisis aguda (acute bee paralysis virus, ABPV), el virus de la parálisis aguda israelí (Israelí acute paralysis virus, IAPV) y el virus Kashmir (Kashmir virus, KBV), así como el complejo del virus de las alas deformadas y el virus *Varroa destructor*-1 (VDV-1). El papel desempeñado por el ácaro en la transmisión de virus puede ser de distintos tipos:

- Vector mecánico: el parásito transmite al virus de abeja a abeja mediante contacto directo, sin que exista una multiplicación del mismo en el ácaro. Este es el caso de los virus pertenecientes al complejo ABPV-IAPV-KBV y el virus SBV (Chen et al., 2004; Di Prisco et al., 2011; Shen et al., 2005).

- Vector biológico: algunos virus de las abejas son capaces de replicarse en el interior del ácaro, de tal modo que cuando la varroa se alimenta de la hemolinfa de las abejas puede "inyectar" gran cantidad de partículas virales tras haberse producido la replicación del virus en el interior del parásito (Ongus et al., 2004; Yue & Genersch, 2005). Cuando esto sucede, las consecuencias pueden ser incluso más devastadoras, ya que, además de la propia inmunosupresión ocasionada por el ácaro, el número de partículas virales es más elevado que en una infección oral. Este es el caso de DWV y VDV-1.

- Activador de infecciones virales persistentes: como se explicará más adelante, muchos virus son capaces de mantenerse en el hospedador en un estado de infección latente persistente. Una infestación por varroa puede ocasionar, debido a su actividad inmunosupresora, una activación de la replicación viral, dando lugar a síntomas e incluso mortalidad. Esto sucede, por ejemplo, con DWV.

## 5.5 Virus

Este punto será abordado en profundidad en el siguiente epígrafe.

## 5.6 El equilibrio de la colmena

Son numerosos los factores implicados en las pérdidas de las colmenas. Sin embargo, un punto en común prevalece y será lo que determine, en muchas ocasiones, que se produzca enfermedad o no. Esto es, el equilibrio dentro de la colmena. Al tratarse de un organismo vivo, debe existir un perfecto equilibrio entre los individuos que la integran. Así, cada abeja se encuentra totalmente especializada en función de su casta y edad, llevando a cabo la actividad correspondiente. La colmena es un organismo vivo en continua evolución, donde la presencia de patógenos es relativamente abundante. Por ello, la salud de las abejas y, en último término, el sistema inmunitario de la colmena, será lo que permita afrontar estas enfermedades, de tal modo que este balance será lo que garantice la supervivencia del conjunto.

Tanto el CCD como la mortalidad invernal son problemas de carácter multifactorial. Ante la presencia de factores de riesgo, alguna de estas causas implicadas puede progresar y producir sintomatología, descenso de la productividad e incluso la muerte de la colmena. El estrés, así como diversos elementos inmunosupresores, pueden ejercer un papel clave a la hora de activar alguno de los factores previamente mencionados. Por ejemplo, un manejo inadecuado por parte del apicultor puede desencadenar un incremento en el nivel de infestación por varroa que, a su vez, puede reactivar infecciones virales. Del mismo modo, puede existir interacción entre diversos factores, como sucede en el caso de las condiciones medio ambientales y el estado nutricional o algunos virus y varroa. A su vez, la presencia de pesticidas puede ocasionar una inmunosupresión subyacente crónica que predisponga a las abejas a sufrir una infección por algún patógeno oportunista. Teniendo en cuenta el elevado número de variables implicadas, el abordaje es complicado y amplio. En los últimos años, la comunidad científica ha llevado a cabo una importante labor de investigación para tratar de elucidar la importancia de cada uno de estos factores, determinándose la creciente necesidad de estudiar del sistema

inmunitario de las abejas y los mecanismos de defensa antivirales (McMenamin et al., 2018), así como de los patógenos, especialmente los virus y varroa (Brutscher, McMenamin, & Flenniken, 2016).

Dentro de los virus que afectan a las abejas, debemos destacar a DWV. Este virus se encontraba presente en las colmenas de abejas de *Apis mellifera* de manera habitual. Sin embargo, con la llegada del ácaro *Varroa destructor* a EEUU y Europa, se produjo un importante cambio en la patogenia del virus, incrementando su nivel de replicación y aumentando su virulencia. Muchos estudios han determinado que este virus, junto con el ácaro, podrían ser buenos predictores del estado de salud de las colmenas (Dainat et al., 2012a), por lo que ambos patógenos requieren especial atención.

# 6. Enfermedades víricas

Dentro del gran número de patógenos que pueden afectar a las abejas, destacan los virus por varias razones. En primer lugar, existe una amplia brecha de información en el estudio de estos patógenos, ya que no se empezaron a describir hasta los años 60. Hasta la fecha, se han descrito 24 virus que pueden afectar a las abejas. Sin embargo, este número es fluctuante y podría incrementarse en los próximos años, ya que las tecnologías de secuenciación de nueva generación están permitiendo un importante avance en el descubrimiento de nuevos virus. Además, los virus son microorganismos habitualmente presentes en las colmenas, aunque en la mayoría de las ocasiones no producen sintomatología y, cuando la originan, ésta suele ser inespecífica y difícil de apreciar. Sin embargo, ante condiciones de estrés e inmunosupresión, las infecciones virales pueden activarse, desencadenando problemas. Por otro lado, los virus de las abejas cuentan con diversas vías de transmisión por las que propagarse, por lo que al entrar en la colmena es muy fácil que se produzca su difusión. Por último, aunque ningún factor ha sido señalado como

el único implicado en las perdidas de las colmenas, los virus han sido asociados con altos ratios de morbilidad y mortalidad en abejas nativas y salvajes (Genersch et al., 2010; Highfield et al., 2009; Schroeder & Martin, 2012; Tehel, Brown, & Paxton, 2016). Los virus juegan, por tanto, un papel clave en las colmenas y deben ser estudiados en profundidad.

## 6.1 Taxonomía y genoma

Los virus se incluyen dentro de la familia *Districtoviridae* (IAPV, KBV, ABPV, BQCV), la familia *Iflaviridae* (DWV, KV, VDV-1, DWV-B, SBV, SBPV), permaneciendo algunos de ellos sin clasificar (chronic bee paralysis virus, CBPV; Lake Sinaí virus, LSVs). Recientemente, se han identificado el virus Bee macula like virus (BeeMLV), perteneciente a la familia *Tymoviridae,* el virus *Apis mellifera* iflavirus y *Apis mellifera* feranovirus (Hartmann et al., 2015; Hou, 2017).

Como ha sido apuntado con anterioridad, han sido descritos 24 virus distintos capaces de infectar a las abejas del género *Apis mellifera* hasta la fecha (Tabla 1). La mayor parte de ellos son virus ARN de cadena sencilla y polaridad positiva, formando partículas icosaédricas de muy pequeño tamaño (17-35 nm), con excepción del virus filamentoso (AmFV) y el virus iridiscente (AIV), cuyo material genético es ADN de doble cadena y tienen un mayor tamaño (150 nm). Desde que en 1963 se descubrió el virus de la parálisis crónica (CBPV) y el virus de la parálisis aguda (ABPV) mediante técnicas clásicas (microscopía o serología), los avances en secuenciación masiva han permitido ampliar la lista de los virus que afectan a las abejas. Algunos virus son muy similares entre sí, por lo que se han agrupado en complejos, como el complejo de la parálisis (formado por ABPV-IAPV-KBV), DWV-VDV-1-Virus Egipcio (EV), SBV-SBV tailandés (TSBV), virus X de la abeja (BVX)-virus Y de la abeja (BYV) y virus del lago Sinaí 1 (LSV-1)-virus del lago Sinaí 2 (LSV-2)-virus del lago Sinaí 3 (LSV-3)-virus del lago Sinaí 4 (LSV-4)-virus del lago Sinaí 5 (LSV-5).

Dicha clasificación taxonómica se basa en la organización del genoma de cada familia, siendo sus principales características las siguientes (Figura 18):

- *Dicistroviridae:* los virus pertenecientes a esta familia se caracterizan por tener dos marcos de lectura abiertos (open reading frame, ORF). El ORF 1 se sitúa en el extremo 5' y codifica para las proteínas no estructurales como helicasa o la polimerasa dependiente de ARN (RdRp), mientras que el ORF 2 codifica para proteínas virales estructurales (VPs). Separando ambos ORF se localiza el Sitio Interno de Entrada al Ribosoma (IRES). El extremo 5' comienza con una proteína enlazada de forma covalente (VPg) y una cola poliA en el extremo 3'.

- *Iflaviridae:* los virus de esta familia tienen el genoma organizado en un único ORF. El extremo 5' codifica para las proteínas estructurales, mientras que el extremo 3' para las proteínas no estructurales. Al igual que en el caso anterior, el extremo 5' comienza con una proteína enlazada de forma covalente (VPg) y una cola poliA en el extremo 3'.

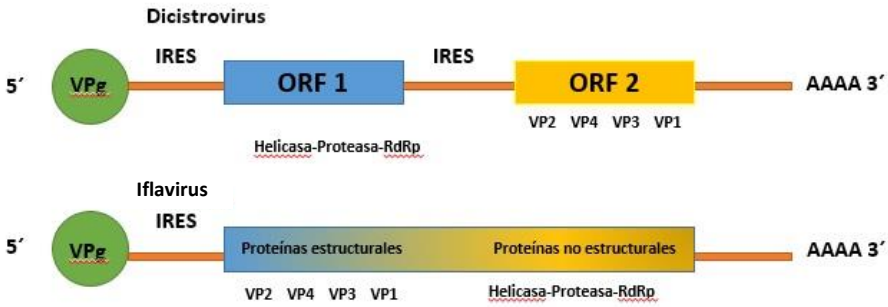


**Figura 18.** Esquema del genoma de los virus de las familias *Dicistroviridae* e *Iflaviridae*. Elaboración propia a partir de Carter y Genersch (2008).

- Virus no clasificados: estos virus cuentan con una estructura del genoma distinta, como por ejemplo CBPV, que tiene siete ORFs distribuidos en dos hebras de ARN.

**Tabla 1.** Características de los virus de abejas. Adaptado de de Miranda et al., 2011. Predicción del genoma. Tamaño de las proteínas calculado mediante SDS-PAGE.

| VIRUS | | PROPIEDADES FÍSICAS | | | | | TAXONOMÍA |
|---|---|---|---|---|---|---|---|
| | | FORMA | TAMAÑO | PROT. CÁPSIDE | ÁC. NUCLEICO | TAMAÑO GENOMA | |
| **Virus de la parálisis aguda** | ABPV | Icosaédrico | 30nm | 35-9-33-24kDa | ssRNA | ~9.5kb | *Dicistroviridae* |
| **Virus Kashmir** | KB | Icosaédrico | 30nm | 37-6-34-25kDa | ssRNA | ~9.5kb | *Dicistroviridae* |
| **Virus de la parálisis aguda israelí** | IAPV | Icosaédrico | 30nm | 35-6-34-25kDa | ssRNA | ~9.5kb | *Dicistroviridae* |
| **Virus de la realera negra** | BQCV | Icosaédrico | 30nm | 31-14-29-30kDa | ssRNA | ~9.5kb | *Dicistroviridae* |
| **Virus de la parálisis letal de los ápidos** | ALPV | Icosaédrico | 30nm | 25-7-32-28kDa* | ssRNA | ~10kb | *Dicistroviridae* |
| **Virus Big Sinoux River** | BSRV | Icosaédrico | 30nm | 28-5-29-30kDa | ssRNA | ~10kb | *Dicistroviridae* |
| **Virus de las alas deformadas** | DWV | Icosaédrico | 30nm | 32-2-44-28kDa | ssRNA | ~10kb | *Iflaviridae* |
| **Virus *Varroa destructor*-1** | VDV-1 | Icosaédrico | 30nm | 32-2-46-28kDa | ssRNA | ~10kb | *Iflaviridae* |
| **Virus egipcio** | EBV | Icosaédrico | 30nm | 30-2-41-25kDa | ssRNA | ? | *Iflaviridae* |
| **Virus de la cría sacciforme** | SBV | Icosaédrico | 30nm | 31-2-32-30kDa | ssRNA | ~9kb | *Iflaviridae* |
| **Virus de la cría sacciforme tailandés/chino** | TSBV | Icosaédrico | 30nm | 31-2-32-30kDa | ssRNA | ~9kb | *Iflaviridae* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Virus de la parálisis lenta** | SBPV | Icosaédrico | 30nm | 27-2-46-29kDa | ssRNA | ~9.5kb | *Iflaviridae* |
| **Virus de la parálisis crónica** | CBPV | Anisométrico | 30-60nm | 23-(30/50/75?)kDa | ssRNA | ~2.3kb/~3.7kb | Sin clasificar |
| **Virus satélite de la parálisis crónica** | CBPSV | Icosaédrico | 17nm | 15kDa | ssRNA | (3x)~1.1kb | Satélite |
| **Virus de las alas opacas** | CWV | Icosaédrico | 17nm | 19kDa | ssRNA | ~1.4kb | ? |
| **Virus X** | BVX | Icosaédrico | 35nm | 52kDa | ssRNA | ? | ? |
| **Virus Y** | BVY | Icosaédrico | 35nm | 50kDa | ssRNA | ? | ? |
| **Virus del lago Sinaí 1** | LSV-1 | ? | ? | 63kDa* | ssRNA | ~5.5kb | Sin clasificar |
| **Virus del lago Sinaí 2** | LSV-2 | ? | ? | 57kDa* | ssRNA | ~5.5kb | Sin clasificar |
| **Virus del lago Sinaí 3** | LSV-3 | ? | ? | ? | ? | ? | ? |
| **Virus del lago Sinaí 4** | LSV-4 | ? | ? | ? | ? | ? | ? |
| **Virus del lago Sinaí 5** | LSV-5 | ? | ? | ? | ? | ? | ? |
| **Virus Virus Arkansas** | ABV | Icosaédrico | 30nm | 43kDa | ssRNA | ~5.6kb | ? |
| **Virus Berkeley similar a picornavirus** | BBPV | Icosaédrico | 30nm | 37-?-35-32kDa | ssRNA | ~9kb | ? |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Virus *Varroa destructor* similar al virus Macula** | VdMLV | Icosaédrico | 30nm | 24kDa* | ssRNA | ~7kb | *Tymoviridae* |
| **Virus filamentoso** | AmFV | Filamentoso | 150x450nm | 12x(13~70kDa) | dsDNA | ? | *Baculoviridae* |
| **Virus iridiscente** | AIV | Poliédrico | 150nm | ? | dsDNA | ? | *Iridoviridae* |

## 6.2 Tipos de infección vírica

Debido a que los virus de las abejas no se replican fácilmente en cultivos celulares, no se puede llevar a cabo el estudio del efecto directo sobre la célula hospedadora. Por tanto, la clasificación clásica de infección lítica, latente y persistente no es posible en los virus de las abejas y se recurre a los términos de "infección aparente" (overt infection) e "infección encubierta" (covert infection).

Cuando se produce una infección de tipo aparente, el hospedador evidencia síntomas de la enfermedad. Este tipo de infecciones se producen si el virus alcanza un alto nivel de replicación, dando lugar a un deterioro del estado físico del hospedador. La transmisión, en este caso, suele ser horizontalmente. Dentro de este tipo de infección diferenciamos, a su vez, dos tipos en función del curso:

- Infección aguda: el hospedador desarrolla una sintomatología muy acusada, en un periodo de tiempo corto. Generalmente se resuelve rápidamente (el hospedador supera la enfermedad o muere) o se convierte en una infección crónica.
- Infección crónica: el desarrollo de la enfermedad es lento, sin un deterioro tan evidente del hospedador. En este caso, el hospedador no es capaz de eliminar el agente, que ocasiona daños a largo plazo.

Por otro lado, cuando se produce una infección de tipo encubierta, el virus se encuentra en los tejidos del hospedador, el cual no desarrolla sintomatología

evidente. El progreso de la enfermedad es lento y dilatado en el tiempo. En este caso, la transmisión se produce de manera vertical durante generaciones y favorece, por tanto, la persistencia de la enfermedad en la población. Pueden desarrollarse signos clínicos en periodos determinados, ante factores estresantes o medio ambientales perjudiciales. Diferenciamos, a su vez, dos tipos:

- Infección encubierta latente: en este caso el virus se encuentra en el interior de las células del hospedador, pero no existe replicación del mismo. Sin embargo, ante factores predisponentes puede producirse activación del virus, dando lugar a replicación del mismo.
- Infección encubierta persistente: el virus se encuentra en los tejidos del hospedador, replicándose a un nivel muy bajo, de tal modo que no se desarrolla sintomatología evidente, pero si se produce un desgaste físico a la larga. En este caso, se alcanza cierto equilibrio entre el virus y el sistema inmunitario del hospedador. De hecho, se ha postulado que este tipo de infección persistente se produce cuando el virus es capaz de evadir los mecanismos defensivos de la abeja (Hails, Ball, & Genersch, 2008). Este tipo de infección es el más frecuente en los virus de las abejas, que persisten en las colmenas replicándose a niveles muy bajos y continuados en el tiempo, lo que ocasiona un desgaste continuo, aunque sin producir destrucción de la célula ni muerte de la abeja. Sin embargo, ante la presencia de factores de riesgo, se puede producir una activación de la replicación viral, desencadenando el desarrollo de enfermedad (Stephenson, 1989).

En general, los virus se encuentran en las colmenas de manera frecuente, produciendo infecciones encubiertas persistentes, sin producir daño evidente en la población, pero generando descensos de la esperanza de vida de las abejas y de su productividad. En muchas ocasiones, las colmenas se hallan infectadas por varios virus a la vez, que pueden o no interactuar entre ellos bajo determinadas circunstancias. Por todo ello, resulta de especial interés el estudio de las dinámicas

poblacionales de los virus dentro de las colmenas de *Apis mellifera*, con el objetivo de determinar la importancia de cada virus y los factores epidemiológicos asociados a cada uno de ellos.

## 6.3 Diagnóstico de enfermedades víricas en abejas

A pesar de que las enfermedades víricas se conocen desde hace siglos, la complejidad de su diagnóstico ha influenciado notablemente en los avances referentes a su diagnóstico. El progreso de las técnicas moleculares ha permitido el desarrollo de una gran variedad de metodologías para el diagnóstico de los virus de las abejas.

Un buen método de diagnóstico debe cumplir las siguientes características:

- Sensibilidad: capacidad para detectar al agente cuando la presencia de éste es mínima, reduciendo así el número de falsos negativos.
- Especificidad: que pueda discernir entre el agente buscado y otros relacionados con el mismo, reduciendo así el número de falsos positivos.
- Robustez: que sea flexible ante posibles variaciones del protocolo y las condiciones en que se realice.
- Simplicidad y rapidez: adaptable a diversos escenarios y proporcionando resultados en el menor tiempo posible.
- Universal: que se pueda realizar en diversos estadios de la enfermedad.
- Barato: coste-beneficio adecuado.

La elección de la técnica, por tanto, debe tener en cuenta todas estas características y será, junto con una adecuada valoración epidemiológica, lo que determinará los resultados obtenidos.

- Características que influyen en el diagnóstico de los virus de las abejas:

Los virus de las abejas presentan una serie de características que dificultan su diagnóstico mediante las técnicas habituales, esto es:

1. La mayor parte de los virus de las abejas son **partículas isométricas**, muy similares entre sí, lo que dificulta el diagnóstico diferencial mediante microscopía electrónica.
2. **No crecen bien en líneas celulares**, a diferencia de otros virus, por lo que no se puede evidenciar el efecto citopático. Recientemente, se han llevado a cabo avances en este campo, utilizándose tecnologías de trasferencia de genes para evadir las limitaciones de los cultivos celulares de abejas (Chan et al., 2010). Sin embargo, se trata de un campo que aún requiere tiempo para desarrollarse.
3. **Las abejas no producen anticuerpos (Ac),** ya que, al igual que otros insectos, carecen de respuesta inmune adaptativa. Por ello, los virus de las abejas no pueden ser detectados por pruebas serológicas indirectas.
4. Dado que varios virus de las abejas pueden coinfectar al mismo individuo, es muy frecuente que se produzcan **infecciones múltiples**. Además, muchos virus de las abejas permanecen en las colmenas sin producir sintomatología, lo que limita en gran medida el diagnóstico mediante ensayos *in vivo.*

- Diagnóstico clásico:

Este tipo de diagnóstico es de tipo cualitativo, a partir de la evidencia de síntomas, partículas víricas o líneas de precipitación. Este tipo de diagnóstico resulta apropiado para las técnicas de manejo que tienen como resultado la implantación o no de un tratamiento. Actualmente, las más empleadas son las siguientes:

1. **Observación de los síntomas:** se trata de una de las principales técnicas de diagnóstico a nivel de campo, ya que es robusta, simple, barata y precisa en el caso de algunas enfermedades víricas. Permite al apicultor evaluar las colmenas, aunque presenta una serie de desventajas. En muchos casos es

imposible visualizar síntomas, dada la capacidad de los virus de las abejas para producir infecciones encubiertas asintomáticas. Además, no siempre se observan síntomas en todos los estadios de la enfermedad ni en todas las edades, lo que dificulta el diagnóstico. Por otro lado, a veces pueden coexistir varias enfermedades, o producirse los mismos síntomas por distintos virus. Es, por tanto, un método de diagnóstico poco sensible y específico, por lo que no permite establecer pautas preventivas y tratamientos adecuados (de Miranda, 2008).

2. **Microscopía electrónica:** es una técnica útil para caracterizar virus en función de su morfología y distribución en tejidos. Sin embargo, en el caso de los virus de las abejas, no resulta eficaz para el diagnóstico, ya que éstos son difíciles de diferenciar morfológicamente.
3. **Ensayos in vivo:** este método consiste en la inoculación de preparados víricos en abejas adultas o larvas para su posterior identificación mediante serología (Dall, 1987). Se trata de una técnica sensible y que puede considerarse semicuantitativa, ya que se pueden realizar diluciones seriadas del inóculo y comparar su detección en pruebas sucesivas. Sin embargo, presenta algunos inconvenientes: es un método que requiere el mantenimiento de las abejas en el laboratorio, así como controlar las condiciones de partida para que los resultados no estén sesgados. Aun así, se trata de un método de gran utilidad para el descubrimiento de nuevos virus y se sigue empleando en la actualidad.
4. **Proteínas y serología:** estas técnicas, basadas en la detección de proteínas víricas en muestras procedentes de abejas, han sido utilizadas para el descubrimiento de distintos virus de las abejas, como son el perfil proteico y la inmunodifusión en gel de agarosa (AGID). Otra técnica empleada fue el ensayo por inmunoabsorción ligado a enzimas (ELISA), que presenta una serie de ventajas (bajo coste, automatizable, permite el análisis de un gran

número de muestras a la vez) pero no permite hacer diagnóstico de múltiples virus de abejas a la vez.

- Diagnóstico molecular mediante PCR:

Actualmente, la técnica más empleada para el diagnóstico de los virus de las abejas es la reacción en cadena de la polimerasa con retrotranscripción (RT-PCR). En un primer momento, se adaptó el diagnóstico de un gran número de virus de abejas mediante RT-PCRs convencionales (de Miranda, 2008), y posteriormente a PCRs a tiempo real (RT-qPCR). Esto último supuso un considerable aumento de la sensibilidad y especificidad en el diagnóstico. Otra ventaja de esta técnica es que permite la realización de un diagnóstico "multiplex" (Schwarz et al., 2013), de tal modo que se pueden identificar distintos virus utilizando el mismo protocolo y placa. Sin embargo, debido al riesgo de falsos positivos, la interpretación de los resultados debe ser cuidadosa.

Además, la PCR en tiempo real permite cuantificar de manera absoluta y relativa la carga viral. Esto resulta de especial interés en el caso de los virus de las abejas, ya que, como ha sido apuntado con anterioridad, se suelen producir infecciones encubiertas en las colmenas y, por tanto, la mera presencia de un virus no es indicativo de enfermedad. En el caso de la **cuantificación relativa**, se utiliza una muestra de referencia a partir de la cual se cuantifican las muestras problema, de tal modo que se compara la carga vírica con respecto a una muestra control. Por el contrario, la **cuantificación absoluta** se realiza mediante el empleo de una curva de calibración o estándar, realizada a partir de un control previamente caracterizado.

## 6.4 Virus de las alas deformadas

El virus de las alas deformadas es un virus perteneciente a la familia *Iflaviridae* que fue aislado por primera vez en Japón en el año 1982, a partir de abejas adultas procedentes de colmenas infestadas por *Varroa destructor*. Tras su descubrimiento, se relacionó con varios casos de muerte de larvas y pupas en

distintos países, así como deformidad de alas en abejas adultas nacidas de colmenas infectadas, lo que le dio su nombre. Inicialmente, sin embargo, fue relacionado con el virus egipcio, por lo que fue denominado como "variante japonesa del EV", aunque posteriormente se comprobó que eran virus distintos (de Miranda & Genersch, 2010; Ribiere, 2008). Se cree que el hospedador original del virus era la abeja asiática, por lo que, al igual que sucedió con el ácaro varroa, podría haber existido un salto inter especie a la *Apis mellifera.* El virus también ha sido detectado en otras especies de abejas silvestres y en abejorros (Genersch et al., 2006). Además, se ha evidenciado su presencia en los parásitos *Tropilaelaps mercedesae* (Dainat et al., 2009) y *Aethina tumida* (Eyer et al., 2009), por lo que éstos podrían actuar como transmisores del virus al parasitar a las abejas.

En ausencia de *Varroa destructor*, la infección por el virus suele mantenerse encubierta, por lo que las abejas no desarrollan síntomas, aunque la presencia del virus puede contribuir a un desgaste energético continuo. Sin embargo, la infestación por varroa puede ser determinante a la hora de incrementar la virulencia del mismo. Los mecanismos que se producen entre el virus y el parásito no son del todo conocidos. La deformidad de alas se evidencia en las fases finales en colmenas predispuestas al colapso, como resultado de un control inadecuado del parásito que permite al virus alcanzar elevados niveles de replicación (Allen & Ball, 1996; Ball, 1993; Bowen-Walker, Martin, & Gunn, 1999; Ribiere, 2008; Sumpter & Martin, 2004b; Tentcheva et al., 2004). Sin embargo, resulta complicado confirmar el papel del virus dentro de las colmenas debido a la dificultad para excluir otros patógenos que podrían estar contribuyendo al colapso de las mismas.

- <u>Genética del virus:</u>

DWV produce partículas icosaédricas formadas por una única cadena de ARN de polaridad positiva y tres proteínas estructurales (Figura 19) (Bailey & Ball, 1991; Lanzi et al., 2006; Ongus et al., 2004), características comunes con el resto de picorna-like virus de los insectos (Moore & Eley, 1991). La organización del genoma es similar

a la del resto de virus de la familia *Iflaviridae*, que consiste en un marco de lectura abierto (ORF) flanqueado por una región 5' (5' UTR) y una corta y altamente conservada región 3' que finaliza en una cola poly-A. Ambas regiones son no traducibles y se encuentran implicadas en la regulación de la replicación y traducción del genoma (Belsham, 2009; Gromeier, Wimmer, & Gorbalenya, 1999; Nakashima & Uchiumi, 2009; Roberts & Groppelli, 2009), lo que resulta en una interacción con los factores del hospedador. En la región 5' UTR se encuentra un IRES, el cual se cree que participa en eludir los mecanismos del hospedador para la traducción del ARN mensajero (Belsham, 2009).

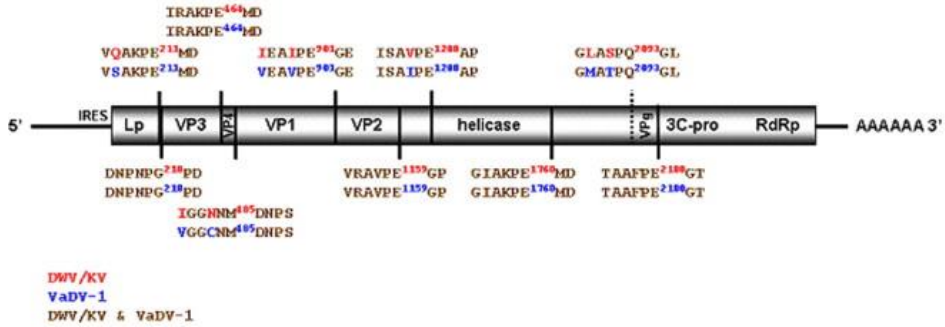


**Figura 19.** Organización del genoma de DWV-KV-VDV-1. Los dominios funcionales identificados son cuatro proteínas de la cápside (VP1-VP4), la helicasa, la proteína viral ligada al genoma (VPG), la 3C-proteasa (3C-pro) y la polimerasa dependiente de ARN. El orden de las proteínas de la cápside es el acordado para la familia Iflavirus. Fuente: Roberts and Gropelli, 2009.

- Variantes del virus:

Al tratarse de un virus ARN, la posibilidad de que se produzcan mutaciones a lo largo de su genoma son muy elevadas. Esto ha favorecido la aparición de mutaciones genéticas que han dado lugar a distintas variantes del virus, así como a cadenas recombinantes. Hasta la fecha, se han descrito tres variantes de DWV: DWV tipo A, también denominada Kakugo Virus (KV), DWV tipo B, también denominada *Varroa destructor* virus-1 y DWV tipo C, una variante aislada recientemente en un

apiario en Swindon, Inglaterra (Mordecai et al., 2016). Las diferencias entre variantes se concentran principalmente en el extremo 5' del genoma y la Lp (Figura 20) (Lanzi et al., 2006). El origen de estas variaciones radica en la ARN polimerasa que, a través de la incorporación de nuevos nucleótidos o bien mediante recombinación con otros virus (o incluso ARN del propio hospedador), puede dar lugar a las mismas durante la replicación (Domingo & Holland, 1997; Roossinck, 1997).

La presencia de una u otra variante puede llegar a tener implicaciones no sólo a nivel molecular, sino también en la virulencia y patogenicidad del virus. Recientes estudios han evidenciado una mayor virulencia por parte de la variante DWV-A/KV, siendo causante del colapso de colmenas. Por otro lado, la variante DWV-B/VDV-1 parece haber alcanzado cierto equilibrio con el hospedador, lo que permite su replicación en niveles elevados sin producir mortalidad (Mordecai, Wilfert, et al., 2016). En contraposición con lo anterior, un estudio llevado a cabo en Estados Unidos puso en duda dicha hipótesis al evidenciar mayor mortalidad en abejas infectadas por la variante DWV-B (Natsopoulou et al., 2017). Sin embargo, dicho estudio fue realizado en condiciones de laboratorio, por lo que las implicaciones reales podrían variar.

- Distribución y estacionalidad:

La distribución de DWV actualmente es mundial. Su estrecha relación con varroa ha propiciado el aumento de su prevalencia en los colmenares de todo el mundo, principalmente desde los años 1970 y 1980 (de Miranda & Genersch, 2010). Su estacionalidad es similar a la del ácaro, aumentando su prevalencia y multiplicación en verano para alcanzar su pico máximo en otoño.

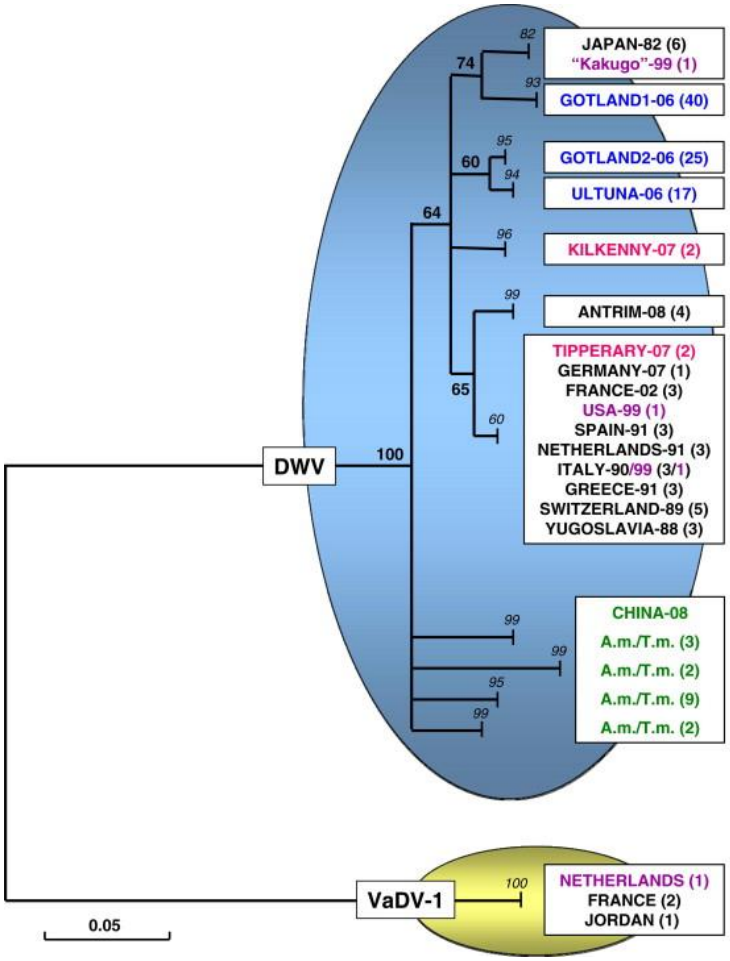


**Figura 20.** Árbol filogenético de los diferentes aislados del virus de las alas deformadas basado en la región Lp del genoma, usando *Varroa destructor* virus (VaDV-1) como otro grupo. Fuente: de Miranda and Genersch, 2010.

- Transmisión y patogenia:

En ausencia de *Varroa destructor,* DWV suele permanecer en las colmenas produciendo infecciones encubiertas persistentes, que se transmiten principalmente por vía vertical (ruta transovárica y a través del semen), aunque también de manera horizontal por la ruta oro-fecal. En este caso, no se suelen evidenciar síntomas y se caracteriza por 1) presencia de partículas virales y ausencia de síntomas, 2)

transmisión vertical y 3) brotes ocasionales de infecciones aparentes como mecanismo para mantener la virulencia (Fievet et al., 2006; Gauthier et al., 2007; Williams et al., 2009; Yue & Genersch, 2005).

En presencia del ácaro, sin embargo, se produce frecuentemente un fenómeno conocido como el síndrome del ácaro parásito: DWV alcanza elevados niveles de replicación dentro del ácaro, el cual, al alimentarse de la hemolinfa de las abejas, les inyecta un gran número de partículas virales. De este modo, es mucho más fácil que se produzcan infecciones sintomáticas, observándose abejas recién nacidas con deformidades, escaso desarrollo de las alas y acortamiento del cuerpo de las abejas (Figura 21). Otros síntomas asociados a la infección de DWV son el acortamiento de la esperanza de vida de las abejas (Martin, 2001), así como cambios en el comportamiento y la capacidad de aprendizaje (Iqbal & Mueller, 2007). También se ha relacionado con un aumento de la agresividad de las abejas, lo que dio nombre a la variante Kakugo virus: dicha variante fue aislada del cerebro de abejas que presentaban comportamientos agresivos, por lo que se utilizó la expresión "kakugo" del japonés, que significa "siempre alerta" y trataba de expresar el comportamiento de agresividad que produce en estos insectos.


**Figura 21.** Abeja con alas deformadas como consecuencia de la infección por DWV. Fuente: Evans, theapiarist.org.

Las consecuencias de la infección por DWV son numerosas (Tabla 2). Por un lado, las abejas que emergen con deformidad en las alas, rápidamente sucumben ante la infección por DWV (Yang & Cox-Foster, 2006). La pérdida prematura de abejas obreras y el desgaste energético constante en las abejas que sobreviven genera un fuerte impacto negativo en el estado de salud de la colmena. Por otro lado, la presencia de infecciones crónicas derivadas de la transmisión vertical de DWV dificulta la recuperación de la colmena.

**Tabla 2**. Tipos de infección producida por DWV en las abejas. Fuente: adaptación a partir de de Miranda, 2010.

| Tipo de infección | Sub-tipo | Síntomas evidentes | Impacto en la colmena | Transmisión vertical | Transmisión horizontal (directa) | Transmisión horizontar (vector) |
|---|---|---|---|---|---|---|
| **Infección aparente** | Aguda | Alas deformadas | Alto | No | No | Sí |
| | Crónica | Desgaste energético | Medio | ? | Sí | Sí |
| **Infección encubierta** | Persistente | Ninguno | Bajo | Sí | Sí | Sí |

- Virulencia del virus:

Resulta imprescindible diferenciar el término virulencia, frecuentemente confundido con patogenia. Para un patógeno dado, la patogenicidad es absoluta y cualitativa, mientras que la virulencia es variable y cuantitativa. La virulencia es, por tanto, la capacidad medible de un patógeno para causar enfermedad, por lo que se asume la existencia de una correlación positiva con el ratio de reproducción del mismo y una correlación negativa con la viabilidad del hospedador (Casadevall & Pirofski, 1999; Sacristan & García-Arenal, 2008; Shaner et al., 1992).

En ausencia del ácaro varroa, DWV es normalmente un virus de baja virulencia, lo que le permite infectar a la cría y que ésta se desarrolle desde el estado de pupa a la adultez (Bailey & Ball, 1991). Debido a esta baja virulencia, DWV es el principal virus asociado al parásito, ya que otros virus de mayor virulencia (CBPV, ABPV, KBV, SBV) matan a la cría rápidamente y no permiten el desarrollo de la pupa (Martin, 2001; Sumpter & Martin, 2004a).

Además de la transmisión dependiente del ácaro, se han descrito otras rutas directas de transmisión horizontal como la alimentación o trophallaxis (trasferencia de alimentos y fluidos entre abejas obreras). Sin embargo, no se ha encontrado relación con un aumento de la virulencia del virus a través de estas rutas.

## 6.5 Otros virus de las abejas

Existe un gran número de virus capaces de infectar a las abejas. A continuación, se destacan los más importantes por su prevalencia y patogenia.

- **Virus de las realeras negras (BQCV):**

Se trata de un virus muy prevalente en España, cuya presencia ha sido relacionada con la del protozoo nosema. Su distribución es mundial, y cuenta con cierta estacionalidad, produciéndose el pico de prevalencia en los meses de verano. La transmisión se produce principalmente mediante vía fecal-oral, aunque también de manera vertical (transmisión mediante esporas de nosema). BQCV es un virus que suele dar lugar a infecciones encubiertas. Cuando produce síntomas, lo cual es poco frecuente, éstos tienen lugar en la cría de reina, y se caracterizan por un oscurecimiento de las realeras y putrefacción de la pupa.

- **Virus de la cría ensacada (SBV):**

La enfermedad de la cría ensacada fue la primera enfermedad de las abejas atribuida a un virus (Bailey, Ball, & Perry, 1983). SBV es un virus ampliamente distribuido, que suele dar lugar a infecciones asintomáticas y su pico se produce en

primavera. La sintomatología se caracteriza por dar lugar a un fallo en la metamorfosis de la cría, produciendo una acumulación de líquido entre la larva y la piel generando un "saco". El color nacarado de la larva se torna amarillento y ésta muere a los pocos días. A pesar de que estos síntomas solo tienen lugar en la cría, SBV puede multiplicarse también en las abejas adultas. Su relación con el acaro varroa ha generado mucha controversia, aunque las últimas evidencias apuntan a que el virus es capaz de multiplicarse en el ácaro y, por tanto, este actúa como vector.

- **Virus de la parálisis aguda israelí (IAPV):**

IAPV es un virus de reciente descubrimiento. De hecho, durante muchos años fue considerado una variante del virus Kashmir. Se trata de un virus de distribución mundial, cuyo pico se produce en verano y otoño, debido a su relación con el ácaro varroa, que actúa como vector del mismo. La transmisión se produce vía fecal-oral, así como de manera vertical y a través de varroa. Normalmente da lugar a infecciones encubiertas en abejas adultas, pero en ocasiones puede generar temblores de alas que se transforman en parálisis, de tal modo que la abeja muere lejos de la colmena durante el pecoreo. Este virus fue directamente relacionado con el SDC por Cox-Foster et al. (2007), en un estudio mediante el cual se evidenció una mayor presencia del virus en colmenas colapsadas. Estudios posteriores, sin embargo, no corroboraron dicha hipótesis (Vicente-Rubiano, Kukielka, de las Heras, & Sánchez-Vizcaíno, 2013).

- **Virus del lago Sinai (LSV):**

Recientes estudios basados en metagenómica han identificado este virus en las abejas de Estados Unidos (Runckel et al., 2011) y, posteriormente, en Bélgica (Ravoet et al., 2013) y España (Granberg et al., 2013). Hasta la fecha, se han identificado 5 variantes distintas del virus (LSV-1, LSV-2, LSV-3, LSV-4 y LSV-5). El papel de este virus en el CCD sigue generando controversia, así como su distribución actual.

- **Virus de parálisis aguda de las abejas (ABPV):**

Este virus fue descrito en 1963 (Bailey & Gibbs, 1964), cuando se investigaba el agente causante de parálisis en abejas. Se trata de uno de los virus más virulentos para las abejas y, aunque su distribución es amplia, su prevalencia es baja. Antes de la llegada de varroa, ABPV daba lugar a infecciones encubiertas principalmente, como resultado de la trasmisión vía fecal-oral o vertical (Yue et al., Schwarz et al., 2013; 2006). Sin embargo, cuando el ácaro inyecta las partículas virales directamente en la hemolinfa, la infección puede dar lugar a sintomatología (parálisis progresiva, oscurecimiento y pérdida de pelo) y muerte (Bailey & Gibbs, 1964).

- **Virus de la parálisis crónica de las abejas (CBPV):**

CBPV fue descubierto junto con ABPV en 1963 (Bailey & Gibbs, 1964), aunque los síntomas producidos por este virus se conocen desde la Antigua Grecia. Al igual que ABPV, es un virus de distribución mundial pero poco prevalente, produciéndose el pico de prevalencia en primavera y verano (Mathieu et al., 2002). Normalmente está presente en las colmenas de manera encubierta, dando lugar a brotes esporádicos de la enfermedad cuando las condiciones de la colmena se ven alteradas, generando dos síndromes distintos: "síndrome tipo 1", caracterizado por parálisis y temblores de alas, y el "síndrome tipo 2", también denominado "ladronas negras", ya que las abejas pierden pelo del abdomen, que se oscurece y adquiere un aspecto brillante, lo que genera rechazo por parte del resto de la colmena al considerarlo pillaje (actividad mediante la cual una abeja roba a otra colmena ajena).

- **Virus Kashmir (KBV):**

Este virus, originario de las abejas asiáticas *Apis cerana,* fue descubierto en 1974 (Bailey & Woods, 1977), afecta también a *Apis mellifera.* Al igual que ABPV y CBPV, se distribuye mundialmente y aumenta su carga en primavera y verano. Se trata de un virus muy virulento con características similares a ABPV, dando lugar a infecciones evidentes con muerte de los individuos cuando se transmite a través de

varroa (Bailey, Carpenter, & Woods, 1979; Ribiere, 2008; Todd, De Miranda, & Ball, 2007).

## 6.6 Infecciones víricas múltiples

Numerosos estudios han evidenciado la presencia de distintos virus en una misma colmena e, incluso, en un mismo individuo (Bacandritsos et al., 2010; Cox-Foster et al., 2007; Genersch et al., 2010; Kukielka et al., 2008; Tentcheva et al., 2004), de tal modo que las infecciones víricas múltiples se consideran muy frecuentes en las abejas. Por tanto, resulta imprescindible estudiar las interacciones y co-infecciones que pueden producirse entre ellos, así como con vectores como varroa. En ocasiones se producen fenómenos de exclusión entre virus, como sucede en el caso de ABPV, que no se replica en pupas que han sido inoculadas con SBV o BQCV (Bailey, Ball, & Perry, 1981).

Estos fenómenos de combinaciones entre virus en un mismo individuo pueden llevar a recombinaciones entre ellos, ya que sus genomas se componen de cadenas simples de ARN, con gran tendencia a las recombinaciones. Por ejemplo, los virus DWV y VDV-1 parecen tener el mismo origen, al igual que sucede en el caso del resto de variantes de DWV (Mordecai, Wilfert, et al., 2016)

# JUSTIFICACIÓN Y OBJETIVOS

# Justificación

Debido a la importante labor que cumplen las abejas como especie polinizadora, su preservación se ha convertido en uno de los principales objetivos de la comunidad científica en la actualidad. El impacto socio-económico y medio ambiental derivado de las pérdidas en sus poblaciones ha fomentado el desarrollo de programas involucrados en su investigación y control. Por ejemplo, en 2012 se inició el "Programa de vigilancia sobre las pérdidas de colonias de abejas" en España, siendo éste el país de la UE con un mayor censo de colmenas, así como un alto nivel de producción de miel. Por todos estos motivos, resulta imprescindible continuar indagando en la materia.

Tras años de investigación, se ha llegado a la conclusión de que las pérdidas de las abejas no están provocadas por una sola causa, sino que existen multitud de factores involucrados, los cuales se interrelacionan entre ellos. Los factores implicados pueden ser tanto bióticos (parásitos, virus, bacterias, hongos) como abióticos (clima, manejo, deficiencias nutricionales, uso de pesticidas y tratamientos acaricidas, entre otros). Entre los factores mencionados, el medio ambiente (lo que determina la disponibilidad de los recursos) y los patógenos se consideran de gran importancia en relación con las pérdidas de las colmenas.

La calidad del paisaje determinará los recursos disponibles para las abejas y, como consecuencia, la calidad de los nutrientes ingeridos, contenidos principalmente en el néctar y en el polen. Una alimentación rica en nutrientes esenciales es fundamental para garantizar el desarrollo adecuado de los individuos de la colmena. Por lo tanto, estudiar las implicaciones del medio ambiente y la

nutrición en las colmenas resulta imprescindible para valorar la importancia de dichos factores.

Dentro de la gran variedad de patógenos que afectan a las abejas, destacan el virus de las alas deformadas (DWV) y el ácaro *Varroa destructor*. Entre ambos agentes se establece una relación sinérgica que puede ocasionar disrupciones en el sistema inmunitario de las abejas y conllevar un desequilibrio dentro de la colmena. Por ello, estudiar la acción conjunta del virus y el parásito en las colmenas de España, así como elucidar los mecanismos implicados en la respuesta inmunitaria frente a estos patógenos, es una tarea imprescindible para mejorar las condiciones sanitarias de las mismas. Además, recientes estudios han evidenciado que, en presencia del ácaro, la virulencia de DWV puede verse incrementada como consecuencia de la aparición de distintas variantes del virus.

Esta tesis doctoral trata, por tanto, de aportar un enfoque integrado que tenga en cuenta la problemática actual de las abejas, centrándose en los pilares involucrados en el mantenimiento del equilibrio dentro de la misma (el medio ambiente, los patógenos y el hospedador).

# Objetivos

El principal objetivo de esta tesis doctoral es determinar el papel y la importancia del virus de las alas deformadas, el ácaro *Varroa destructor* y los factores medio ambientales en el desencadenamiento de pérdidas en las colmenas, así como sus implicaciones en el sistema inmunitario de las abejas.

Los objetivos específicos de esta tesis doctoral son:

**Objetivo 1.** Determinar la relación entre la variabilidad genética del virus de las alas deformadas y la virulencia del mismo.

**Objetivo 2.** Desarrollar potenciales marcadores del sistema inmunitario de las abejas en relación con la presencia del virus de las alas deformadas y el ácaro *Varroa destructor*.

**Objetivo 3.** Determinar la importancia del medio ambiente y la nutrición en la salud de las colmenas:

**Sub objetivo 3.1** Determinar la importancia de la diversidad del polen ingerido por las abejas en relación a los virus y *Varroa destructor.*

**Sub objetivo 3.2** Determinar la importancia de las localizaciones de los colmenares en relación a la salud de las colmenas.

**Objetivo 4.** Analizar la relación del virus de las alas deformadas, el virus de las realeras negras, el virus de la cría ensacada, el virus de la parálisis aguda israelí y el ácaro *Varroa destructor* con la fortaleza de las colmenas en el sur de España.

# Chapter 1

## Assessment of the relationship between the genetic variability of deformed wing virus and its virulence

# Objective 1

## Assessment of the relationship between the genetic variability of deformed wing virus and its virulence

**Article published in a peer reviewed journal**

- **S. Barroso-Arévalo,** M. Vicente-Rubiano, F. Puerta, F. Molero, J.M. Sánchez-Vizcaíno. "Nucleotide sequence variations may be associated with virulence of deformed wing virus". Apidologie. DOI:10.1007/s13592-019-00660-5

**Proceedings**

- **S. Barroso-Arévalo**, J. Goyache Goñi, F. Molero del Rosal; F. Puerta Puerta, J.M. Sánchez-Vizcaíno Rodríguez. "Virulencia del virus de las alas deformadas: la secuencia sí importa". In V Jornadas de Investigación en Doctorado (VETINDOC), Universidad Complutense de Madrid. Facultad de Veterinaria, Universidad Complutense de Madrid (June 2019). Oral communication.

# CHAPTER 1

# Resumen

Las abejas son una de las principales especies polinizadoras de la cual dependen multitud de cultivos. En los últimos años, estos insectos se han visto amenazados por muchos factores, lo que se ha reflejado en un descenso de sus poblaciones y en un incremento de la mortalidad invernal. En particular, la entrada del ácaro *Varroa destructor* en Estados Unidos y Europa ha conllevado importantes cambios en la epidemiología y patogenia del virus de las alas deformadas. Entre ambos agentes se establece una relación sinérgica, ya que la varroa actúa como vector biológico del virus. Además de aumentar la tasa de transmisión del virus, la replicación dentro del ácaro puede conllevar la selección genética de distintas variantes del virus, el cual tiene una elevada capacidad de mutación. Hasta la fecha, se han descrito tres variantes diferentes de DWV: variante tipo A (también conocida como Kakugo virus, KV), variante tipo B (también denominada *Varroa destructor* virus 1, VDV-1) y variante tipo C. Existe controversia acerca de la virulencia relativa de cada variante.

En el estudio descrito en el presente capítulo, se seleccionaron dos colmenas de *Apis mellifera:* colmena H (Healthy, sana) y colmena W (Weak, débil). Se analizó la presencia y carga de DWV y el porcentaje de infestación por el ácaro *Varroa destructor* en un periodo de 21 meses. Una muestra de cada colmena fue amplificada mediante la técnica Whole Transcriptome Amplification (WTA), secuenciada y analizada mediante el empleo de técnicas filogenéticas. La secuencia procedente de la colmena H, la cual presentó un adecuado estado de salud a lo largo de todo el estudio, mostró mayor semejanza con la secuencia descrita para DWV-B, aunque presentó recombinaciones con la variante DWV-A. Esta secuencia se agrupó con las variantes DWV-B disponibles en el GenBank, que pertenecían a colmenas sanas. La colmena W, sin embargo, presentó sintomatología de alas deformadas y mortalidad a lo largo del

periodo de estudio. La secuencia de esta colmena fue similar a la variante DWV-A y se agrupó con secuencias pertenecientes a colmenas con síntomas y mortalidad.

Dado que los análisis habían sido realizados en tan solo dos muestras, se decidió comprobar estos resultados en otras colmenas pertenecientes al colmenar experimental. Para ello, se aplicó el análisis ABC (Kevill, Highfield, Mordecai, Martin, & Schroeder, 2017) para la determinación mediante qPCR de la variante predominante en otras colmenas del colmenar, así como en un mayor número de muestras en las colmenas H y W. Dichos resultados apoyaron la veracidad de los hallazgos previos, ya que aquellas colmenas con peor estado sanitario (sintomatología de alas deformadas, menor población, menor cantidad de cría y producción) presentaron, principalmente, infección por la variante tipo A. En cambio, aquellas colmenas con mejor estado sanitario solían ser positivas y tenían cargas más elevadas de la variante tipo B. DWV-C, en cambio, no fue detectado en ninguna de las muestras analizadas, lo que sugiere que no se trata de una variante ampliamente distribuida en España, al menos en el norte peninsular.

Por lo tanto, este trabajo ha determinado la existencia de al menos dos variantes de DWV (DWV-A y DWV-B) en el sur de España. Además, los resultados obtenidos sugieren que las variaciones a nivel genético en la secuencia del virus pueden estar implicadas en la virulencia del mismo, lo que podría modificar las medidas de control en las colmenas.

# Abstract

Western honey bees (*Apis mellifera*) are key players in crop pollination and in the maintenance of global biodiversity. Their viability is threatened by *V. destructor*, which acts as a vector of the deformed wing virus (DWV). Several genetic DWV variants have been reported, but it is unclear whether their virulence differs.

We examined the prevalence of *V. destructor* and DWV as well as bee health in two colonies over 21 months, and then characterizing DWV variants from each colony using phylogenetics. Colony H showed no signs of disease or mortality, and DWV sequence from this colony clustered with VDV/DWV-B sequences previously reported in healthy colonies. Colony W showed DWV symptoms, and DWV sequence clustered with DWV-A sequences previously reported in colonies with symptoms.

These results suggest that nucleotide variations in the DWV genome can affect its virulence. Genotyping DWV variants in colonies may be an effective tool to assess risk and initiate preventive measures early.

# CHAPTER 1

# 1.1 Introduction

Deformed wing virus (DWV), a single-stranded, positive-sense RNA virus of the genus *Iflavirus* (Genersch & Aubert, 2010; Ongus et al., 2004), has become one of the most challenging honey bee pathogens. Historically, DWV did not represent a serious threat to honey bee colonies because it could persist as a covert infection without causing apparent symptoms. However, when carried by the globally prevalent ectoparasitic mite *Varroa destructor* (Wilfert et al., 2016), infection easily becomes overt and the honey bees can show wing deformity, shortened abdomen and reduced life span (de Miranda & Genersch, 2010). The combination of *V. destructor* and DWV has contributed to substantial death of honey bee colonies worldwide (Guzmán-Novoa et al., 2010; Martin et al., 2012; C. E. Thompson, Biesmeijer, Allnutt, Pietravalle, & Budge, 2014). DWV is capable of replicating within the mite, in such a way that *V. destructor* acts as a biological vector (Martin, 2001; Shen, Yang, Cox-Foster, & Cui, 2005). In addition to transmitting viruses such as DWV, the mite externally digest and consume fat body tissue (Ramsey et al., 2019), and feeds on honey bee haemolymph, causing weight loss at individual level (Yang & Cox-Foster, 2005). At colony level, the mite renders the colony more vulnerable to viral infection and leads to lower vitellogenin levels, which can reduce survival during overwintering (Amdam et al., 2004).

The mite appears to have created a particularly effective mode of DWV transmission because the mite's feeding behavior means that DWV infects honey bees at the pupal stage, which is more likely to lead to viral loads $>10^{10}$ Genome Equivalent Copy/bee that are usually associated with morphological DWV symptoms (Gisder, Aumeier, & Genersch, 2009; Möckel, Gisder, & Genersch, 2011). Recent studies have shown that *V. destructor* has introduced a new viral transmission route which has transformed the viral landscape, dramatically decreasing DWV diversity (Martin et al., 2012).. Recent studies have linked these viral landscape changes with a selection of a virulent recombinant strain of DWV denominated as DWV-A, which

replicates to high levels in honey bees only when directly inoculated into haemolymph by *V. destructor* or experimental injection (Kevill et al., 2017; E. A. Mordecai, L. Wilfert, et al., 2016; Ryabov et al., 2014). This virulent recombinant form of DWV may predispose developing larvae and pupae to developing deformed wing symptoms and, consequently, reducing productivity and their life expectancy. For example, a study carried out by Mordecai et al. (2016) proposed a phenomenon known as superinfection exclusion in honey bees from Swindon, UK (Mordecai et al., 2016). Since its discovery, DWV-A has been sub classified into two types, DWV (Lanzi et al., 2006) and Kakugo virus (KV) (Fujiyuki et al., 2004). According to demarcation criteria, DWV-B, originally called VDV-1 (Mordecai, Brettell, et al., 2016), shows ~84% nucleotide identity to DWV-A and has been shown to replicate in *V. destructor* and honey bees (Ongus et al., 2004; Zioni, Soroker, & Chejanovsky, 2011). Recently, Mordecai et al. (2016) have reported the DWV-C, which is a third established variant that has been reported to contribute to winter colony losses (Kevill et al., 2017). It also suggested that the DWV type C has not recently emerged, but also is an established DWV variant. However, the high virus´ ability to mutate and recombine difficult the genomic analysis of the sequences.

Several previous studies have established that genomic variations in DWV can affect tropism, pathogenicity and epidemiology (Gisder et al., Gisder, Mockel, Eisenhardt, & Genersch, 2018; Möckel et al., 2011). This implies that genetic analysis of DWV in honey bee colonies may allow the early identification of colonies at risk of damaging infection and timely implementation of preventive measures. Data on the prevalence of DWV variants in Spain is lacking, although government data suggest the presence of the virus in 83% of colonies and 99% of apiaries (MAPA, 2017).

In the present study, we identified two DWV variants in southern Spain and examined whether genomic differences between them may influence their virulence. DWV load and *V. destructor* infestation were monitored in 10 colonies of an

experimental apiary in Andalusia, Spain over a 21-month period. Of the ten colonies evaluated, two of them were selected based on the following criteria: health status, viral load and *V. destructor* infestation. One DWV-positive sample from each selected colony was sequenced, and the results were compared with complete DWV genomes sequences from the GenBank. Additionally, a follow up study based on RT-qPCR of the three DWV master variants (ABC assay) (Kevill et al., 2017) was performed. Therefore, the main objective of this study was to determine if the virus sequence was related to the virus virulence. On the remaining colonies, ABC assay (Kevill et al., 2017) was also applied in order to evaluate the distribution of the DWV master variants on the apiary. A secondary objective was to determine what variants of the DWV are present in the South of Spain, which is one of the most important Autonomous Community regarding beekeeping.

# 1.2 Material and methods

## 1.2.1 Bee sampling and colony health determination

Bees were collected from 10 colonies of an experimental apiary at the Reference Centre for Beekeeping, University of Cordoba, Spain. All colonies were similarly managed. These colonies were studied from March 2015 to January 2017, except for July and August 2015, when sampling was not possible. Colonies were treated against *V. destructor* using Apitraz® in March and September. Samples of adult bees were taken from the hive entrance of each colony, frozen at -80 °C until analysis, and then analysed for DWV load and *V. destructor* infestation level. A total of 149 samples were collected.

Every month, the beekeeping technician inspected all colonies and determined the number of bee, brood, pollen and honey combs, as well as the presence of DWV symptoms. Colonies with signs of poor population (low activity in

the entrance of the colony) or fewer than five bee and brood combs were categorized as having a "poor population". Otherwise, colonies were categorized as having an "adequate population" if they showed high activity level in the entrance of the colony and more than 6-7 frames covered with bees and 2-3 frames covered with capped brood, considering the beekeeping managing and the time of the year. Health-related events were also recorded in the colonies, such as viral symptoms (deformity in wings and nervous symptoms), symptoms of bacterial disease and mortality.

### 1.2.2 DWV load determination

DWV load was determined in samples by homogenising 10 whole bees with mortar and pestle in 5 ml of phosphate-buffered saline (PBS). This amount of starting material should allow detection of DWV if present in more than 25% of bees with a detection probability of 99% at the colony level (Pirk et al., 2013). RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and stored at −80 ◦C. This RNA served as template in one-step real-time reverse transcription polymerase chain reaction based on SYBR Green detection as described (Kukielka, Esperón, Higes, & Sánchez-Vizcaíno, 2008).

Two colonies were selected based on health status, *V. destructor* infestation level, and viral load, which was classified into the four infection categories defined by (Amirir et al., 2015). Health status was defined based on the population size, as assessed from the number of bee/honey/pollen/brood combs, on the presence of DWV symptoms (deformed wings, shortened and rounded abdomens, paralysis), and mortality. Colony H (healthy colony) showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as described below. Colony W (weak colony) showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis.

### 1.2.3 *Varroa destructor* determination

*V. destructor* load was quantified in all colonies throughout the study except for July and August 2015. Mite presence was assessed at each monthly sampling. Mite load was quantified using the soapy water method described in "Standard methods for varroa research" in the COLOSS BEEBOOK (Dietemann et al., 2012).

### 1.2.4 Whole-transcriptome amplification

Total RNA was extracted from the Colony H and Colony W samples mentioned above and were selected to perform the phylogenetic analysis after amplification using TransPlex Whole Transcriptome Amplification (SIGMA-ALDRICH). This kit provides a rapid method for preparing amplified cDNA from total RNA for downstream RNASeq applications. It employs a single primer isothermal amplification (SPIA) method to amplify total RNA into double stranded cDNA and depletes rRNA without preselecting mRNA. In brief, sample homogenate (600 µl) was centrifuged for 10 min at 1792 g, the supernatant was passed through at 0.45-µm membrane filter, and RNA was isolated using the column-based Nucleospin II Virus® kit (Macherey-Nagel) following the manufacturer's instructions. Total RNA was quantified using the Nanodrop system (Thermo Fisher Scientific, Wilmington, US) and amplified as cDNA using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, San Luis, US) according to the manufacturer's instructions, except that each reaction contained 3 µl of template RNA and the amplification involved 30 cycles. Each sample was split into three and processed in parallel. Amplified cDNA was purified using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's protocol. Concentration of amplified cDNA was measured using the Nanodrop (128–297 ng/µl), and the ratio of absorbance at 260/280 and 260/230 nm (Table 1). A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA; expected 260/230 values are commonly in the range of 2.0-2.2. Therefore, the obtained values indicated reasonably pure DNA (Wilfinger, Mackey, & Chomczynski, 1997).

**Table 1.** Amounts and purity assessment of cDNA after whole-transcriptome amplification**.**

| Sample | Amount (ng/ µl) | 280/260 ratio | 230/260 ratio |
|---|---|---|---|
| **Colony H-1** | 170 | 1.74 | 2.15 |
| **Colony H-2** | 159.4 | 1.81 | 1.9 |
| **Colony H-3** | 177.5 | 1.86 | 2.15 |
| **Colony W-1** | 214.8 | 1.89 | 2.26 |
| **Colony W-2** | 202.3 | 1.80 | 1.58 |
| **Colony W-3** | 297.1 | 1.84 | 2.10 |

## 1.2.5 PCR amplification and DNA sequencing

The DWV genomes in the samples from Colony H and Colony W were sequenced nearly completely using the primer walking approach with 48 PCR primer pairs (Supplementary data 1), which were designed based on the genomes of DWV (NC_004830.2) and VDV-1 (AY251269.2). PCR was performed using the high-fidelity PrimeSTAR® HS DNA Polymerase (Takara, Saint-Germain-en-Laye, France) in reactions (25 µl) containing 15 µl of 5x PrimeSTAR Buffer, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of cDNA template and 6 µl of RNAse- and DNase-free water. Reactions were subjected to 35 cycles of denaturation at 94 °C for 2 min, annealing at 94 °C for 30 sec, and extension at 70 °C for 5 min in a T3000 thermocycler (Biometra, Göttingen, Germany).

The amplified PCR products were analysed using 1% agarose gel electrophoresis in 45 mM Tris borate (pH 8.0), 2.5 mM EDTA (0.53 TBE) containing 0.5 mg/ml ethidium bromide; DNA products were visualized by transillumination with a long-wave UV light box. PCR products were purified using a PCR Purification Kit (Qiagen, Germantown, USA) and >700 bp (excluding primers) were sequenced

using the Sanger method on an ABI Prism 3730 (Applied Biosystems, Foster City, CA, USA). The sequencing primers were the same as those used for amplification.

Colony H and Colony W sequences were edited and assembled into nearly complete DWV genomes using MEGA 6 software (Tamura et al., 2013). These sequences were aligned with published sequences using ClustalW. One alignment contained 16 complete genomes of DWV, KV and VDV (Supplementary data 2), together with the sequences from Colony H and Colony W. Another alignment was based on the variable RNA-dependent RNA polymerase (RdRp) region in 39 DWV genomes, included the sequences from Colony H and Colony W. Alignments were edited by hand where necessary based on conserved protein domains as a guide. The two final alignments were considered adequate because the first was associated with an average amino acid p-distance (1 - amino acid identity) of 0.074, and the second, with an average p-distance of 0.069. These values are within the acceptance threshold of <0.8 (Thompson et al., 1999; Ogden & Rosenberg, 2006) (Ogden and Rosenberg 2006). From these alignments, phylogenetic trees were constructed using the maximum likelihood method and SPR algorithm and bootstrap testing of 2000 replicates.

The genomes sequenced from Colony H and Colony W were analysed for recombination using the Recombination Detection Program 4.1 (Martin et al. 2015), with its algorithms GENECONV (Padidam et al., 1999), BootScan (Martin et al. 2005), MaxChi (Smith, 1992), CHIMAERA (Posada & Crandall, 2001), SISCan (Gibbs et al., 2000), and 3Seq (Boni et al., 2007). Default parameters and Bonferroni correction for multiple comparisons were used. $P < 0.05$ was regarded as statistically significant. Only recombination events that more than four algorithms identified as statistically significant were included in further analysis.

### 1.2.6 RT-qPCR ABC Assay

For further investigation to the DWV strains distribution in Colony H and Colony W, as well as within the remaining colonies of the study, ABC assay (Kevill et al., 2017) was performed. In the case of Colony H and Colony W, all samples collected after the sampling used for sequencing were evaluated using ABC assay. In the remaining colonies, three samples from each colony were analysed, according to the following criteria: if the colony died during the study (called Dx), samples from the three last samplings before collapsing were selected. If the colony did not die during the study (called Sx), samples from the last three samplings were selected.

One-step, real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), SYBR Green detection, cycling protocols and primers for DWV-A, DWV-B and DWV-C previously published (Kevill et al., 2017). Cycling protocol was slightly amended: RT step occurred at 45 º C for 15 min and annealing at 61 ºC for 15 s.

Load of positive samples was determined by absolute quantification based on a standard curve constructed using serial 10-fold dilutions of known amounts of PGemT® TA plasmid (Promega, Madison, USA) containing the target genes (RdRp region) of DWV master variants type A, B and C. Standard curves were fitted with lines showing correlation coefficients of 0.99 (data not shown).

## 1.3 Results

### 1.3.1 DWV and *V. destructor* loads in selected samples

Colony H showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as

described above. This sample showed a DWV load of 1.70 x $10^9$ GEC and *V. destructor* infestation level of 10.68%.

Colony W showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis. This sample showed DWV load of 6.70 x $10^7$ GEC and *V. destructor* infestation of 1.45%.

### 1.3.2 Colony health during the study

Two DWV genomes from colonies in southern Spain were sequenced for this study. Both genomes were 9,031 nt long, excluding 3' poly-adenylated tails (GenBank accession MK262742 and MK262743). One genome came from a colony with good health status, reflected in high population and adequate numbers of bee/honey/pollen/brood combs. This Colony H showed high DWV load in 10 of 21 monthly samplings. *V. destructor* infestation rate was higher at the beginning and end of the study (Figure 1). Despite high DWV and mite levels, the beekeeping technician reported the population to be adequate and stable throughout the study period. Anti-mite treatments successfully decreased mite levels and DWV load. Pearson correlation analysis showed a positive correlation between the two pathogens ($r = 0.591$, $p = 0.015$). No DWV symptoms were detected in this colony throughout the study.

The second genome came from a colony (Colony W) that presented deformed wings, mortality and smaller population than the other colonies in the apiary. In March 2015, near the beginning of the study, the colony showed high DWV load, which decreased slightly over time (Figure 2). *V. destructor* was present from the beginning of the study but decreased after anti-mite treatment. Toward the end of the study, viral load and *V. destructor* infestation rate increased. This colony died in November 2016, two months before the end of the study. Before death, the colony

showed high DWV load (2.4 x $10^7$ GECs), small population and the DWV symptom of deformed wings.

In both colonies, *V. destructor* infestation rate and, to a lesser extent, DWV load varied seasonally, with pathogen levels highest during the summer and at the beginning of the fall. Both colonies showed high levels of both pathogens but responded differently to them.

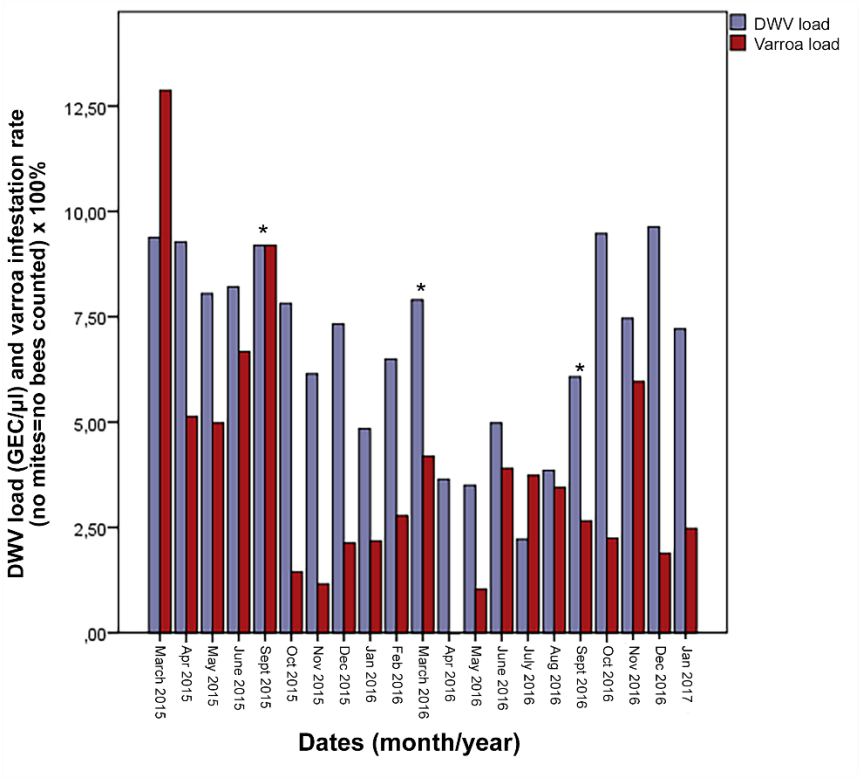


**Figure 1.** DWV load and V. destructor infestation rate in Colony H during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

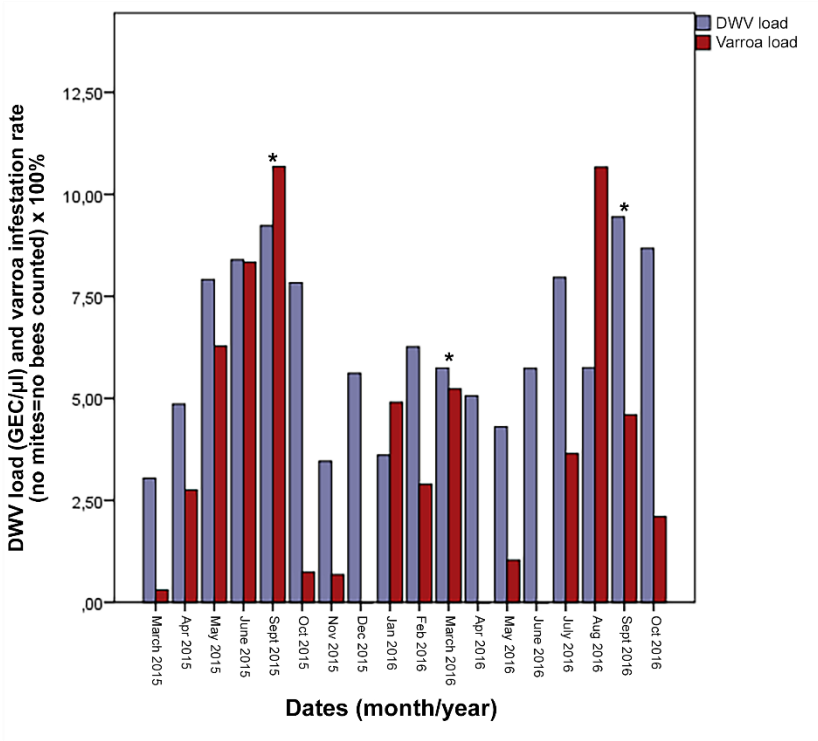


**Figure 2.** DWV load and V. destructor infestation rate in Colony W during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

### 1.3.3 Phylogenetic analysis based on complete DWV genome sequences

Specific primers pairs were applied to both DWV positive colonies in order to sequence the complete DWV genome (Supplementary data 1). The complete DWV genome sequences from Colony H and Colony W were aligned with the 16 complete genomes of DWV or VDV-1 available in GenBank (Supplementary data 2). The alignment was 9,045 nt long, corresponding to genome sequences determined here (9,031 nt) as well as some gaps. The sequences determined here covered 88.5% of the DWV reference genome (NC_004830.2). Alignment of both sequences showed 7,088 of 9,031 nt (78.5%) to be identical. The nucleotides showed low divergence among the DWV reference genomes used for the alignment (Table 2).

**Table 2.** Matrix of percent identity from alignment of DWV variants. Colors are used to indicate percent identity: dark blue, >95%; light blue, 90–95%; orange, 85-90%; yellow, 80-85%.

| Sequence | DWV type A | VDV-1 | VDV-1/DWV | DWV Colony H | DWV Colony W |
|---|---|---|---|---|---|
| DWV type A | 100% | 84% | 92.2% | 97.19% | 97.19% |
| VDV-1 | 84% | 100% | 90.2% | 97% | 83.85% |
| VDV-1/DWV | 92.2% | 90.2% | 100% | 88.5% | 91,79% |
| DWV Colony H | 85.3% | 97% | 88.5% | 100% | 85.1% |
| DWV Colony W | 97.19% | 83.85% | 91.79% | 85.1% | 100% |

2,117 variable nucleotide positions were identified across the entire genome. Among segregating sites, the average pairwise nucleotide diversity between sequences was $\pi = 0.073$, and the Tajima D test statistics rejected the neutrality hypothesis ($D = 0.146$). The Colony H genome exhibited 85.3% similarity to the DWV-A reference genome (NC_004830.2), 97% similarity to the VDV-1 reference genome (AY251269.2) and 88.5% similarity to the recombinant VDV-1/DWV genome (KX373900.1). The corresponding similarity percentages for the Colony W genome were 89% similarity to the DWV type A, 77.1% to the VDV-1 and 84% to the VDV-1/DWV recombinant (Figure 3).

Evolutionary relationships among DWV genomes were inferred using maximum likelihood based on the general time-reversible model (Nei & Kumar, 2000). The analysis involved 18 nucleotide sequences, including 1st, 2nd, 3rd and noncoding codon positions. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. In the end, 9,031 positions were analysed. The bootstrap consensus tree inferred from 2000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). Branches were collapsed if the corresponding partitions occurred in fewer than 50% of bootstrap replicates. Initial tree(s) for the heuristic search were obtained

automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using maximum composite likelihood, and then selecting the topology with better log likelihood value. Differences in rate of evolution among different sites were modeled using a discrete gamma distribution (2 categories, +G, parameter = 0.2136).

Phylogenetic analysis showed that the Colony H genome clustered with genomes identified from honey bee colonies without DWV symptoms. These other genomes included one from an experimental apiary in Belgium, the VDV-1 reference genome, and two recombinant VDV-1/DWV genomes from the UK. In contrast, the Colony W genome clustered with genomes from colonies with DWV symptoms, mainly deformed wings (Supplementary data 2). The most closely related sequence was from an Austrian colony with losses.

### 1.3.4 Recombination breakpoints

We examined recombination events in the entire dataset of complete genomes, without any assumption of putative parental sequences. A total of 17 recombination events were detected (Supplementary data 3). This analysis suggested that the Colony H genome showed three recombination events. The first recombination event (between VDV-1/DWV-B and DWV-A) between a genome from Belgium (JX783225.1), which came from a colony that lacked DWV-specific symptoms but showed relatively short lifespan, and the DWV reference genome (NC_004830.2), which came from a colony that had deformed wings. It was located at 1045-1685 nt, encoding the VP2 and VP3. The second recombination (VDV-1/DWV-B and DWV-A) was located at 4163-4703 nt, encoding the VP3 and the helicase. The third recombination event (between DWV-A and DWV-A) was located at 8895-9026 nt, encoding the N-terminal region.

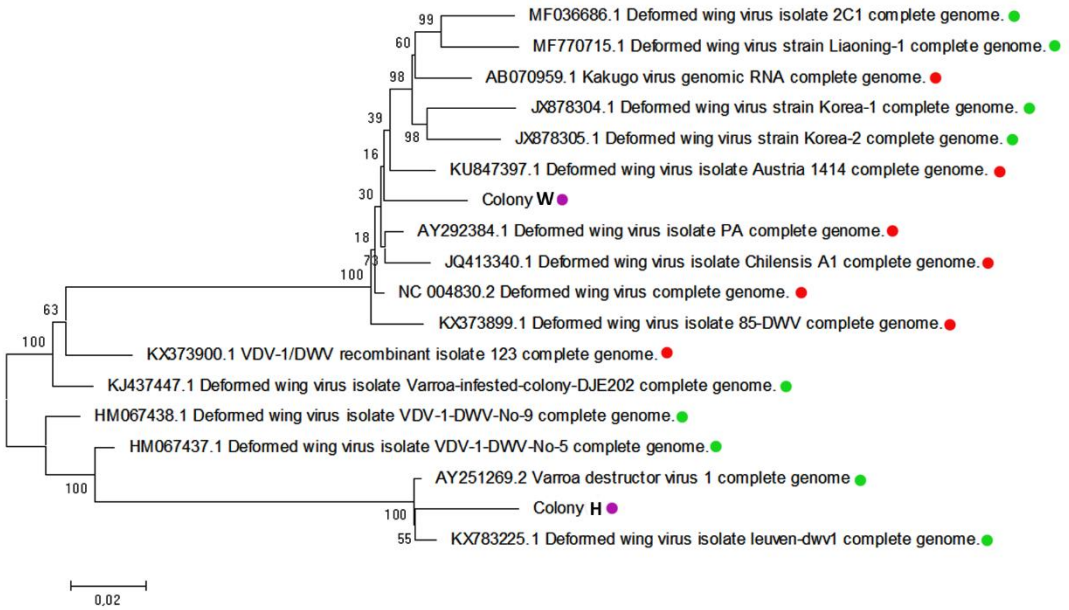


**Figure 3.** Maximum likelihood phylogenetic analysis of complete DWV genomes. A total of 16 complete DWV, VDV–1 and KV genome sequences were obtained from the GenBank database and aligned with the two nearly complete genomes reported here. Red circles indicate previously published sequences from colonies with symptoms (deformed wings, mortality, low population); green circles, previously published sequences from colonies without any symptoms. Purple circles mark the two sequences obtained in the present study.

The analysis suggested that the Colony W genome showed a recombination event between a genome from INRA in Avignon, France (KX373899.1), which came from a colony that showed deformed wings, and a genome from South Korea (JX878304.1), which came from a colony that lacked DWV-specific symptoms.

### 1.3.5 Phylogenetic analysis based on RdRp regions of the DWV genome

In order to explore phylogenetic diversity based on the variable RdRp region (nucleotide position 9,265-9594 in the DWV reference complete genome), we

performed a second phylogenetic tree. Analysis based on 37 DWV RdRp sequences in Genbank showed a cluster including DWV and KV genomes and another including VDV-1 genomes. The Colony W genome fell within the DWV-KV cluster, while the Colony H genome fell within the VDV-1 cluster (Figure 4).

### 1.3.6 Detection of DWV master variants using the ABC assay

In Colony H, ABC assay revealed that DWV-B was the most prevalent variant, although DWV-A was also present, but to a lesser extent. DWV-B load was higher than DWV-A load in this colony. In Colony W, DWV-A was detected in all the samples, meanwhile DWV-B was detected on a smaller number of samples and at lower load (Supplementary data 4).

In the remaining colonies, ABC assay was applied to the three months before collapsing (if the colony died) or to the three months before the end of the study (if the colony did not died). Five of the eight remaining colonies died before the end of the study period (Supplementary data 5). RT-qPCR results (ABC assay) showed that DWV-A and DWV-B variants were detected in honey bee samples selected from the other eight colonies, meanwhile DWV-C was not detected in any sample. DWV type A was detected in the majority of samples tested, being the dominant variant in colonies that collapsed. On the other hand, DWV type B was also quite prevalent and achieved higher load in surviving colonies.

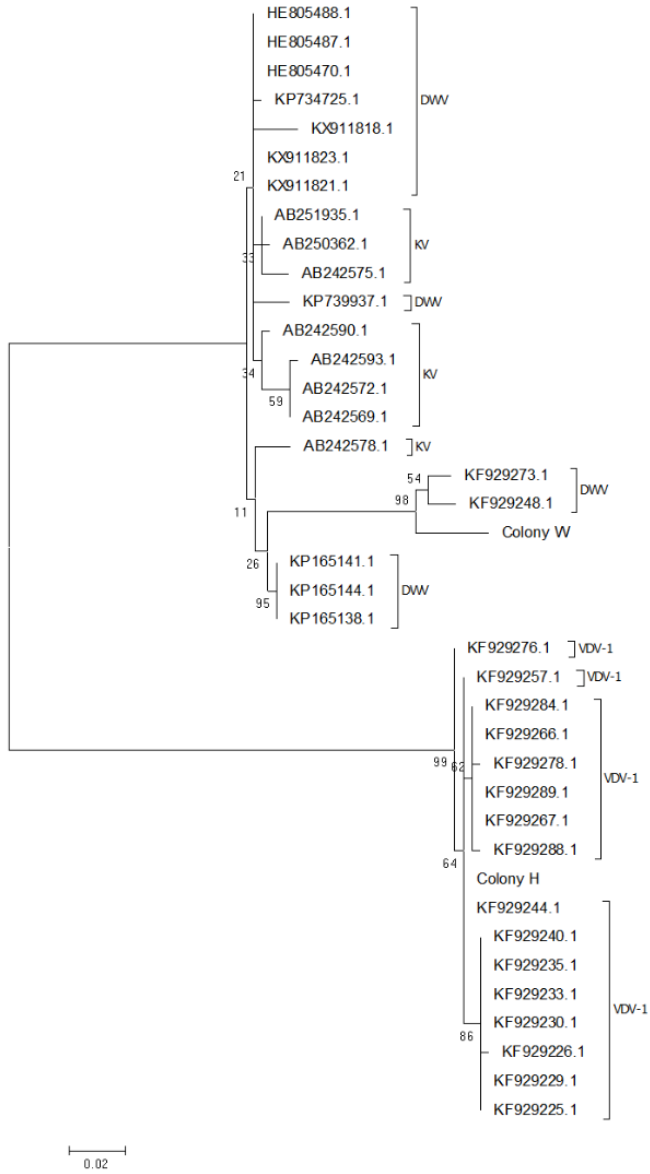


**Figure 4.** Maximum likelihood phylogenetic analysis based on the variable RdRp region in the DWV genome. A total of 35 RdRp sequences from DWV, VDV–1 and KV in GenBank were compared with the two RdRp sequences determined here.

# 1.5 Discussion

Positive-strand ssRNA viruses are associated with honey bee colony losses; in particular, the combination of DWV and *V. destructor* has been related to strong virulence and severely colony mortality (B. Dainat, J. D. Evans, Y. Chen, L. Gauthier, & P. Neumann, 2012b). Field evidence have demonstrated that the presence of DWV and *V. destructor* is an important factor contributing to the current colony losses, but how both pathogens interact so effectively to trigger colony losses requires further study. Phylogenetics suggests that the mite may be driving changes in the DWV genome (Mordecai, Wilfert, et al., 2016; Mordecai et al., 2016; Ryabov et al., 2014), but it is unclear whether such genetic changes affect viral virulence and therefore risk of colony loss (McMahon et al., 2016; Natsopoulou et al., 2017). The present study assessed whether nucleotide variations in the DWV genome can influence virulence. Our results suggest that different DWV variants at the same load can trigger different effects on colonies in the same apiary that are exposed to the same conditions and show similar *V. destructor* infestation. These findings suggest that nucleotide differences in the DWV strain can make the difference between colony health and collapse, implying that DWV genotyping of colonies may facilitate early identification of colonies at greater risk of collapse. At the same time, our study provides the first nearly complete sequences of DWV isolates from southern Spain, where the beekeeping sector is more professional than in other parts of the country.

We were able to sequence nearly the entire DWV genomes from the two colonies in our study, including the internal ribosome entry site, the variable RdRp sequence and the sequences encoding the L protein, structural proteins, and helicase. We were unable to sequence two open reading frames of 949 and 161 nt, flanked respectively by the 5' or 3' non-translated region, or the poly(A) tail. The Colony H genome (accession number MK262742), from a healthy colony, showed recombination at three breakpoints between the DWV reference genome from Italy (Lanzi et al., 2006) and a DWV sequence from Belgium (Benaets et al., 2017). The

Colony H genome clustered with sequences that mainly came from healthy colonies, including the VDV-1 reference genome (97% similarity) and recombinant VDV-1/DWV genomes from the UK (Moore et al., 2011). Despite the fact that the Colony H genome mainly clustered with VDV-1/DWV-B genomes, it was a recombinant between VDV-1/DWV-B and DWV-A. Therefore, both DWV-A and B wild-type genomes must have been present simultaneously. For this to be the case, the DWV-A genome has had to diverge significantly enough to mainly cluster with VDV-1/DWV-B and not with the other DWV-A/DWV-B recombinants. It showed three recombination events throughout its genome. The first recombination event was located in a similar region of the genome as reported by Mordecai et al (Mordecai, Brettell, et al., 2016), between the Lp and the VP2. However, the second recombination event was located in the VP3 and the helicase region. Therefore, it is, to our knowledge, the first report of a recombination between DWV-B and DWV-A in this region of the genome. The Colony W genome (accession number MK262743), from a weak colony with DWV symptoms, showed one recombination event between the same sequence from Belgium and an isolate from South Korea (Reddy et al., 2013). The Colony W genome clustered with sequences from colonies with symptoms and mortality. It also clustered with the KV reference genome. Similar clustering results for the Colony H and Colony W isolates were obtained in phylogenetics analyses based only on the variable RdRp sequence.

Despite not all the nucleotides of the DWV sequences from the Spanish apiary were sequenced, our primer walking approach covered part of the IRES, L protein and the structural proteins, as well as the helicase and the RdRp, in order to study recombination events in these regions of the viral genome. These results show that substantial genetic variation can be found in DWV strains affecting the same apiary. Indeed, the isolates from Colony H and Colony W showed only 85.5% similarity. This variation may help explain differences in viral virulence (Mordecai, Brettell, et al., 2016; Mordecai, Wilfert, et al., 2016) and therefore colony outcomes such as in the present study. Our results are consistent with previous reports that

VDV-1, DWV-B and their recombinants are less virulent than DWV-A and KV (Mordecai, Brettell, et al., 2016). Our findings justify further work into the potential association between DWV nucleotide sequence and virulence.

While differences in DWV virulence on their own may explain why Colony H survived with good health despite high viral load and *V. destructor* infestation rate, another potential factor is so-called superinfection exclusion (Folimonova, 2012). According to this concept, an established virus infection can interfere with secondary infection by the same or a closely related virus. This exclusion may benefit the established virus because it reduces competition for resources and stabilizes its genome by reducing the risk of recombination with other viral genomes in the same cell. This exclusion may help explain how the mite can decrease DWV diversity by making certain DWV variants dominate over others (Mordecai, Brettell, et al., 2016). Superinfection exclusion may protect the honey bee colony if an established infection with a weakly virulent DWV strain (e.g. transmitted by the mite) prevents secondary infection with virulent DWV-A. Under the assumption that DWV-B can be regarded as weakly virulent, and taking into account that Colony H genome was a DWV-B/DWV-A recombinant, our results provide the first supporting evidence of superinfection exclusion at the colony level by a recombinant DWV-A/DWV-B (more similar to DWV-B). One of the issues that emerges from this finding is if the region of the genome where recombination occurs play a key role in determining DWV virulence, since the recombinant DWV from Colony H protected the colony. This should be also carefully confirmed in light of reports that DWV-B may correlated with losses in worker number over the overwintering period, as it was suggested by a recent study carried out by Natsopoulou et al. (Natsopoulou et al., 2017). McMahon et al. also probed, under laboratory conditions, that DWV-B could be more virulent than the DWV-A variant (McMahon et al., 2016). In this experiment, the authors observed higher mortality in the DWV-B infected group, compared to the mock-infected control group. However, these experiments need to be replicated in honey bees from different geographical locations, at different viral loads, and under field

conditions to test this conclusion. All of these variables make it difficult to understand the current role of variant DWV-B.

When exploring RdRp region by constructing a phylogenetic tree, sequence from Colony H clustered with VDV-1 sequences and sequence from Colony W clustered with DWV and KV sequences. These results confirmed the similarity between the sequences from this study and the stipulated DWV variants.

ABC assay on Colony H and W revealed that the distribution of DWV-A and DWV-B variants was relatively constant over the study period. DWV-A was detected in both colonies, but high DWV-B loads may have protected Colony H from DWV-A, since DWV-A was present at lower levels and this colony survived the study period and did not show any symptoms. On the contrary, Colony W showed higher DWV-A loads and lower DWV-B loads, which may have contributed to its collapse. DWV-A titles over $10^7$ GEC were detected in Colony W the months before collapsing, meanwhile DWV-B was not detected in some samplings or it was present at low levels. This result may be explained by the fact that DWV-B was not in sufficiently high levels to protect the colony from the virulent DWV-A.

Additionally, ABC assay on colonies that died revealed that DWV-A was the dominant variant in the three months before collapsing. By contrast, colonies that survived showed lower DWV-A load and higher DWV-B load, which could have had a protective effect on those colonies. These findings are consistent with those of other studies (Kevill et al., 2017; Martin et al., 2012; Mordecai, Brettell, et al., 2016; Schroeder & Martin, 2012) and support the hypothesis that DWV-A can be more virulent but high DWV-B titles may have a protective effect on colonies. However, these data must be interpreted with caution because of the low sample size, and further study of the potential protective effect of DWV-B should be done. Surprisingly, DWV-C was not detected in any sample, which may reveal that this variant is not established in Southern Spain yet. Therefore, DWV-C implications on colony health could not be assessed.

Our study indicates the presence of at least two DWV strains in southern Spain: VDV-1/DWV-B recombinant with DWV-A (more similar to DWV-B) (DWV-SpB), and DWV-A (DWV-SpA). These findings extend our limited understanding of the global distribution of DWV variants (Robert Scott Cornman et al., 2013; Kielmanowicz et al., 2015). Further work, which consider these results, should examine the prevalence of variants in Spain and more broadly in Europe, since Europe has been reported to be a critical source for the global spread of the DWV variants.

Recombination breakpoints were identified in the Colony H genome at nt 1095-1685 in the regions encoding VP2 and VP1, as well as at nt 4163-4703 in the region encoding the VP3 and the helicase. These two breakpoints were derived from the DWV-A reference sequence. A third breakpoint was identified at nt 8895-9026 in the N-terminal region, derived from the DWV sequence from Belgium. The Colony W genome showed a recombination breakpoint at nt 3828-4408 overlapping with the region encoding the helicase and derived from a South Korean isolate showing deformed wing symptoms. These results clearly show that honey bee colonies can be infected by a mixture of DWV and its recombinants, which may result from multiple modes of virus transmission.

In this study, we report the nearly complete genome sequences of two DWV isolates from an apiary in southern Spain and show that they correspond to two DWV variants. We show evidence of genetic diversity in DWV populations and for an association between genomic sequence and viral virulence. Genetic changes in DWV may help it adapt to its host and its vector (*V. destructor*). In conclussion, our findings add evidence to a growing body of literature on the genetic study of DWV, and emphasize the importance of understanding pathogen genetic diversity when investigating causes of honey bee losses.

# Supplementary data

**Supplementary data 1.** Primers used for sequencing based on primer walking.

| Primer pairs | Oligo name | Sequence | Product size |
|---|---|---|---|
| WTA-1 Common | WTA-1 Fw | GGCACCCGTTAATGTCTCAT | 703 bp |
| | WTA-1 Rev | GCMCKAACRACTTTYTCRCG | |
| WTA-2 Common | WTA-2 Fw | GCGCGTGATAATGAGTTCCT | 784 bp |
| | WTA-2 Rev | CACGCCAATATGCATGTACC | |
| WTA-3 Common | WTA-3 Fw | GATGATCCATTTGATAAGGA | 650 bp |
| | WTA-3 Rev | GGGACAAAATGACGAGGAGA | |
| WTA-4 Common | WTA-4 Fw | CAAATCAGGGCAAAACCTG | 627 bp |
| | WTA-4 Rev | CACATAGGGCACTTCAAAMG | |
| WTA-5 Colony H | WTA-5 H Fw | CGGTGAGCGTAAAATGGAGT | 754 bp |
| | WTA-5 H Rev | GGGACAAAATGACGAGGAGA | |
| WTA-6 Colony H | WTA-6 H Fw | TGTTCCTGGACTGACTGCTTC | 836 bp |
| | WTA-6 H Rev | ATTCTTCGCCTTCTGGAACA | |
| WTA-7 Colony H | WTA-7 H Fw | AAGGACCCGGCAAAGTAAGT | 817 bp |
| | WTA-7 H Rev | TGCGAAAACTGCATACCATC | |
| WTA-8 Colony H | WTA-8 H Fw | GGTTGGTTTTCAGGCCACTA | 796 bp |
| | WTA-8 H Rev | CGCTGCAGGATTACTCTGAC | |
| WTA-9 Colony H | WTA-9 H Fw | CGCTCAAAAACCGAAACAAT | 805 bp |
| | WTA-9 H Rev | ACGATCAAACCTCGGAAAAG | |
| WTA-10 Colony H | WTA-10 H Fw | ATGCAACGAGCTCTTACGTG | 897 bp |
| | WTA-10 H Rev | TGTTCATGCGCTTCAATTTC | |
| WTA-11 Colony H | WTA-11 H Fw | ACAACGTGTTGGCTCAGAGA | 786 bp |
| | WTA-11 H Rev | AAACGCCATGTTGGATTTTC | |
| WTA-12 Colony H | WTA-12 H Fw | TTTGGTGCCAGAAGGAGACT | 706 bp |
| | WTA-12 H Rev | ATGTAATGCCGCAACATCAA | |
| WTA-13 Colony H | WTA-13 H Fw | GACGCCGCTGTGAATAAGAT | 796 bp |
| | WTA-13 H Rev | CGATCATACGGTTCCCTTTC | |
| WTA-14 Colony H | WTA-14 H Fw | TGGTGTTGCTGAACCTCTTG | 902 bp |
| | WTA-14 H Rev | TATGCGTGCCATACTTCGAC | |
| WTA-15 Colony H | WTA-15 H Fw | TCCCGTCCAATTTACGATTC | 832 bp |
| | WTA-15 H Rev | AAAGTATTCGGGACCCCATC | |
| WTA-16 | WTA-16 H Fw | GAGGATTGGGTCGTCGAGTA | 455 bp |

| | | | |
|---|---|---|---|
| Colony H | WTA-16 H Rev | GCGACAATACGCGAGTAACA | |
| WTA-5 | WTA-5 W Fw | GCTTTGATTAGTGCGTCAGC | 785 bp |
| Colony W | WTA-5 W Rev | TGCAAAGATGCTGTCAAACC | |
| WTA-6 | WTA-6 W Fw | TAATCGCTCCCTTACGGATG | 619 bp |
| Colony W | WTA-6 W Rev | CCGACAATTAACCTGCCAGT | |
| WTA-7 | WTA-7 W Fw | AATGGCGTGGTGCATTAGA | 834 bp |
| Colony W | WTA-7 W Rev | TGACTTCCCATACCGGATTT | |
| WTA-8 | WTA-8 W Fw | CTGATGAGAAGGCCAAACAA | 728 bp |
| Colony W | WTA-8 W Rev | AGCGCTAGACGCATTAAACG | |
| WTA-9 | WTA-9 W Fw | CATTCAAATCACGCGTGTAAA | 780 bp |
| Colony W | WTA-9 W Rev | GGTTTTTGAGCAGCCACATT | |
| WTA-10 | WTA-10 W Fw | CCGGATCTTCAACAACTGGA | 820 bp |
| Colony W | WTA-10 W Rev | CAATTCCAGATGCACCACAC | |
| WTA-11 | WTA-11 W Fw | CAATTTGAGATTGCGTCGAG | 840 bp |
| Colony W | WTA-11 W Rev | AACGATTCATTCGCCTTACG | |
| WTA-12 | WTA-12 W Fw | CATGGTCTGAATGGATGACG | 656 bp |
| Colony W | WTA-12 W Rev | TCATCCGTAGAAAGCCGAGT | |
| WTA-13 | WTA-13 W Fw | CTGCCGACTTTGATCTTGTG | 723 bp |
| Colony W | WTA-13 W Rev | CTTGAGGTGATGCCAGTCCT | |
| WTA-14 | WTA-13 W Fw | TCCGAAACCAACTTCTGAGG | 813 bp |
| Colony W | WTA-13 W Rev | AAGCCATGCAATCCTTCAGT | |
| WTA-15 | WTA-14 W Fw | GTCCCGTGAAAATGATTCGT | 787 bp |
| Colony W | WTA-14 W Rev | AACTCCAAAGCCATGCAATC | |
| WTA-16 | WTA-15 W Fw | CTGAAGGATTGCATGGCTTT | 834 bp |
| Colony W | WTA-15 W Rev | GTCGTGCAGCTCGATAGGAT | |
| WTA-17 | WTA-16 W Fw | CGACGCAGTTAATGAGGAAA | 766 bp |
| Colony W | WTA-16 W Rev | GTCTGTAACGTCCGCCACTT | |
| WTA-18 | WTA-17 W Fw | CTTGTCTGTTATGGCGACGA | 712 bp |
| Colony W | WTA-17 W Rev | CGCGAGTAACACCTAACCTAGA | |

**Supplementary data 2.** Complete DWV genomes used to generate the whole-genome phylogenetic tree. Information regarding the presence of DWV symptoms was recovered from the associated publications.

| Ascension number | Authors | Geographic origin | Length (nt) | Definition | Associated symptoms |
|---|---|---|---|---|---|
| JQ413340.1 | Barriga,G.P. et al. | Chile | 10171 | Deformed wing virus isolate Chilensis A1, complete genome. | Deformed wings |
| AY292384.1 | Lanzi,G. et al, | Italy | 10166 | Deformed wing virus isolate PA, complete genome. | Deformed wings |
| KX373899.1 | Dalmon,A. et al. | France | 10149 | Deformed wing virus isolate 85-DWV, complete genome. | Deformed wings |
| NC_004830.2 | Lanzi,G. et al. | Italy | 10140 | Deformed wing virus, complete genome. | Deformed wings |
| KU847397.1 | Lamp,B. et al. | Austria | 10203 | Deformed wing virus isolate Austria 1414, complete genome. | Colony losses |
| JX878305.1 | Reddy,K.E. et al. | Korea | 10114 | Deformed wing virus strain Korea-2, complete genome. | No symptoms |
| JX878304.1 | Reddy,K.E. et al. | Korea | 10111 | Deformed wing virus strain Korea-2, complete genome. | No symptoms |
| AB070959.1 | Fujiyuki,T. et al. | Japan | 10152 | Kakugo virus genomic RNA, complete genome. | Aggressiveness |
| MF770715.1 | Fei,D., et al. | China | 10167 | Deformed wing virus strain Liaoning-1, complete genome. | No symptoms |
| MF036686.1 | Wu,Y., et al. | China | 9838 | Deformed wing virus isolate 2C1, complete genome. | No symptoms |
| KX373900.1 | Dalmon,A. et al. | France | 10112 | VDV-1/DWV recombinant isolate 123, complete genome. | Deformed wings |
| KJ437447.1 | Moore, J. et al. | United Kingdom | 10167 | Deformed wing virus isolate Varroa-infested-colony-DJE202, complete | No symptoms |
| HM067438.1 | Moore, J. et al. | United Kingdom | 10154 | Deformed wing virus isolate VDV-1-DWV-No-9, complete genome. | No symptoms |
| HM067437.1 | Moore, J. et al. | United Kingdom | 10149 | Deformed wing virus isolate VDV-1-DWV-No-5, complete genome. | No symptoms |
| KX783225.1 | Benaets,K. et al. | Belgium | 10112 | Deformed wing virus isolate leuven-dwv1, complete genome. | No symptoms |
| AY251269.2 | Ongus,J.R. et al. | The Netherlands | 10142 | *Varroa destructor* virus 1 complete genome | No symptoms |

**Supplementary data 3**. Recombination events in the DWV genome. Events were detected across the entire genome in the dataset of 16 previously published complete genome sequences and 2 nearly complete sequences determined here. The highest acceptable p value was 0.05. Algorithms are indicated as follows: R, Recombination Detection Program; G, GENECONV; B, Bootscan; M, MaxChi; C, Chimaera; S, SiScan; T, 3Seq.

| Isolate | Major parent | Minor parent | Breakpoint localizations | Method | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | R | G | B | M | C | S | T |
| KX373900.1 | AY292384 | AY251269.2 | 807-4768 | + | + | + | + | + | + | + |
| KJ437447.1 | NC_004830.2 | AY251269.2 | 737-4165 | + | + | + | + | + | + | + |
| HM067438.1 | AY292384 | AY251269.2 | 4205-9044 | + | + | + | + | + | + | + |
| HM067438.1 | AB070959.1 | KX783225.1 | 1-4150 | + | + | + | + | + | + | + |
| Colony H | AY251269.2 | NC_004830.2_D | 1095-1685 | + | + | + | + | + | + | + |
| HM067438.1 | KJ437447.1 | AY251269.2 | 4873-9039 | + | + | - | + | + | + | - |
| Colony H | AY251269.2 | NC_004830.2 | 4163-4703 | + | + | + | + | + | + | + |
| HM067438.1 | KX373900.1 | HM067437.1 | 8418-9023 | + | + | + | + | + | - | + |
| Colony H | KX783225.1_De | JX878304.1_De | 8895-9026 | + | + | + | + | + | + | + |
| KJ437447.1 | NC_004830.2 | JX878304.1 | 7923-8697 | + | - | + | + | + | + | + |
| AB070959.1 | Unknown | AY292384 | 3755-4857 | + | + | + | + | + | + | + |
| Colony W | KX373899.1 | JX878304.1 | 3828-4408 | + | + | + | + | + | + | + |
| KJ437447.1 | AY292384 | HM067437.1 | 6025-7924 | + | + | + | + | + | + | + |
| AB070959.1 | NC_004830.2 | MF770715.1 | 6982-9033 | + | - | + | + | + | - | + |
| JX878305.1 | NC_004830.2 | MF770715.1 | 7957-9027 | + | - | + | + | + | - | + |
| JX878304.1 | NC_004830.2 | MF770715.1 | 7885-9023 | + | - | + | + | + | + | + |
| AY292384 | NC_004830.2 | KU847397.1 | 6871-7545 | + | - | - | + | + | - | + |
| HM067437.1 | NC_004830.2 | MF036686.1 | 8717-9010 | + | - | + | + | + | - | + |

**Supplementary data 5.** ABC assay (Kevill et al., 2017) on colonies that were not sequenced. Analyses were performed on samples from the three months before collapsing (if the colony collapsed) or before ending the study (if the colony died not collapse). DWV variant type and viral loads are showed for each sample. Darker shades indicate higher viral load. $D_x$ indicates colonies that died, and $S_x$ colonies that survived.

| Colony ID | Collection Date | DWV-A | DWV-B | DWV-C | Colony health |
|---|---|---|---|---|---|
| D1 | March 2015 | $3{,}05 \times 10^8$ | - | - | Rapid collapse. Low population. High *V. destructor* load |
| | April 2015 | $1{,}62 \times 10^8$ | - | - | |
| | May 2015 | Collapse | Collapse | - | |
| D2 | March 2015 | $2{,}73 \times 10^9$ | $6{,}94 \times 10^4$ | - | Rapid collapse. Low population. Low *V. destructor* load |
| | April 2015 | $1{,}09 \times 10^8$ | $3{,}29 \times 10^3$ | - | |
| | May 2015 | $4{,}10 \times 10^7$ | - | - | |
| D3 | April 2015 | - | $6{,}67 \times 10^5$ | | Collapse probably due to high temperatures |
| | May 2015 | $7{,}26 \times 10^2$ | $2{,}36 \times 10^2$ | | |
| | June 2015 | $5{,}31 \times 10^5$ | $2{,}20 \times 10^6$ | | |
| D4 | February 2016 | $4{,}04 \times 10^3$ | $2{,}98 \times 10^4$ | - | Overwinter losses. High *V. destructor* load |
| | March 2016 | $3{,}82 \times 10^6$ | $5{,}37 \times 10^7$ | - | |
| | April 2016 | $1{,}32 \times 10^7$ | $6{,}12 \times 10^5$ | - | |
| D5 | September 2016 | $1{,}18 \times 10^6$ | - | - | Low population and DWV symptoms before collapsing |
| | October 2016 | $1{,}54 \times 10^7$ | $5{,}03 \times 10^3$ | - | |
| | November 2016 | $2{,}40 \times 10^7$ | - | - | |
| S1 | November 2016 | - | $2{,}55 \times 10^4$ | - | High population. No symptoms |
| | December 2016 | - | $9{,}65 \times 10^6$ | - | |
| | January 2017 | - | $4{,}13 \times 10^5$ | - | |
| S2 | November 2016 | $6{,}89 \times 10^4$ | $4{,}11 \times 10^8$ | - | High *V. destructor* load. No symptoms |
| | December 2016 | $1{,}66 \times 10^7$ | $3{,}90 \times 10^6$ | - | |
| | January 2017 | $2{,}59 \times 10^2$ | $7{,}97 \times 10^7$ | - | |
| S3 | November 2016 | $9{,}59 \times 10^6$ | $4{,}43 \times 10^8$ | - | High population. No symptoms |
| | December 2016 | $2{,}09 \times 10^3$ | $9{,}35 \times 10^9$ | - | |
| | January 2017 | - | $5{,}29 \times 10^8$ | - | |

**Supplementary data 4**. ABC assay (Kevill et al., 2017) on Colony W and H after sequencing. DWV variant type and viral loads are shown for each sample. Darker shades indicate higher viral load.

| Colony ID | Colony H | | | Colony W | | |
|---|---|---|---|---|---|---|
| Collection date | DWV-A | DWV-B | DWV-C | DWV-A | DWV-B | DWV-C |
| October 2015 | $3{,}67 \times 10^{2}$ | $1{,}38 \times 10^{6}$ | - | Not evaluated | | |
| November 2015 | $4{,}90 \times 10^{3}$ | $2{,}14 \times 10^{7}$ | - | $4{,}17 \times 10^{4}$ | - | - |
| December 2015 | $3{,}32 \times 10^{5}$ | $6{,}92 \times 10^{4}$ | - | $4{,}07 \times 10^{2}$ | $2{,}37 \times 10^{3}$ | - |
| January 2016 | - | $3{,}16 \times 10^{8}$ | - | $1{,}86 \times 10^{6}$ | - | - |
| February 2016 | - | $7{,}94 \times 10^{7}$ | - | $4{,}63 \times 10^{5}$ | - | - |
| March 2016 | - | $4{,}37 \times 10^{3}$ | - | $1{,}15 \times 10^{5}$ | $9{,}13 \times 10^{2}$ | - |
| April 2016 | $9{,}87 \times 10^{2}$ | $3{,}16 \times 10^{3}$ | - | $2{,}00 \times 10^{4}$ | $3{,}07 \times 10^{4}$ | - |
| May 2016 | - | $9{,}55 \times 10^{4}$ | - | $5{,}37 \times 10^{5}$ | $1{,}03 \times 10^{2}$ | - |
| June 2016 | - | $1{,}66 \times 10^{2}$ | - | $9{,}12 \times 10^{8}$ | - | - |
| July 2016 | $1{,}94 \times 10^{2}$ | $7{,}08 \times 10^{3}$ | - | $5{,}62 \times 10^{7}$ | $9{,}98 \times 10^{2}$ | - |
| August 2016 | - | $1{,}17 \times 10^{6}$ | - | $2{,}82 \times 10^{9}$ | - | - |
| September 2016 | - | $2{,}95 \times 10^{9}$ | - | $4{,}79 \times 10^{9}$ | $1{,}34 \times 10^{5}$ | - |
| October 2016 | $1{,}08 \times 10^{3}$ | $2{,}88 \times 10^{7}$ | - | $2{,}40 \times 10^{7}$ | $6{,}49 \times 10^{3}$ | - |
| November 2016 | $2{,}52 \times 10^{4}$ | $4{,}27 \times 10^{9}$ | - | Collapse | | |
| December 2016 | $9{,}40 \times 10^{2}$ | $1{,}62 \times 10^{7}$ | - | | | |

# CHAPTER 1

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**Original article**

# Nucleotide sequence variations may be associated with virulence of deformed wing virus

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**Abstract** – Western honey bees (*Apis mellifera*) are key players in crop pollination and in the maintenance of global biodiversity. Their viability is threatened by *Varroa destructor*, which acts as a vector of the deformed wing virus (DWV). Several genetic DWV variants have been reported, but it is unclear whether their virulence differs. We examined the prevalence of *V. destructor* and DWV as well as bee health in two colonies over 21 months and then characterizing DWV variants from each colony using phylogenetics. Colony H showed no signs of disease or mortality, and DWV sequence from this colony clustered with VDV/DWV-B sequences previously reported in healthy colonies. Colony W showed DWV symptoms, and DWV sequence clustered with DWV-A sequences previously reported in colonies with symptoms. These results suggest that nucleotide variations in the DWV genome can affect its virulence. Genotyping DWV variants in colonies may be an effective tool to assess risk and initiate preventive measures early.

**deformed wing virus / *V. destructor* / virus virulence / phylogeny / honey bee**

## 1. INTRODUCTION

Deformed wing virus (DWV), a single-stranded, positive-sense RNA virus of the genus *Iflavirus* (Genersch and Aubert 2010; Ongus et al. 2004), has become one of the most challenging honey bee pathogens. Historically, DWV did not represent a serious threat to honey bee colonies because it could persist as a covert infection without causing apparent symptoms. However, when carried by the globally prevalent ectoparasitic mite *Varroa destructor* (Wilfert et al. 2016), infection easily becomes overt and the honey bees can show wing deformity, shortened abdomen, and reduced life span (de Miranda and Genersch 2010). The combination of *V. destructor* and DWV has contributed to substantial death of honey bee colonies worldwide (Guzmán-Novoa et al. 2010; Martin et al. 2012; Thompson et al. 2014). DWV is capable of replicating within the mite, in such a way that *V. destructor* acts as a biological vector (Martin 2001; Shen et al. 2005). In addition to transmitting viruses such as DWV, the mites externally digest and consume fat body tissue (Ramsey et al. 2019) and feed on honey bee hemolymph, causing weight loss at individual level (Yang and Cox-Foster 2005). At colony level, the mite renders the colony more vulnerable to viral infection and leads to lower vitellogenin levels, which can reduce survival during overwintering (Amdam et al. 2004).

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The mite appears to have created a particularly effective mode of DWV transmission because the mite's feeding behavior means that DWV infects honey bees at the pupal stage, which is more likely to lead to viral loads > $10^{10}$ genome equivalent copy/bee that are usually associated with morphological DWV symptoms (Gisder et al. 2009; Möckel et al. 2011). Recent studies have shown that *V. destructor* has introduced a new viral transmission route which has transformed the viral landscape, dramatically decreasing DWV diversity (Martin et al. 2012). Recent studies have linked these viral landscape changes with a selection of a virulent recombinant strain of DWV denominated as DWV-A, which replicates to high levels in honey bees only when directly inoculated into hemolymph by *V. destructor* or experimental injection (Kevill et al. 2017; Mordecai et al. 2016b; Ryabov et al. 2014). This virulent recombinant form of DWV may predispose developing larvae and pupae to developing deformed wing symptoms and, consequently, reducing productivity and their life expectancy. For example, a study carried out by Mordecai et al. (2016a) proposed a phenomenon known as superinfection exclusion in honey bees from Swindon, UK (Mordecai et al. 2016a). Since its discovery, DWV-A has been sub-classified into two types, DWV (Lanzi et al. 2006) and Kakugo virus (KV) (Fujiyuki et al. 2004). According to demarcation criteria, DWV-B, originally called VDV-1 (Mordecai et al. 2016a), shows ~ 84% nucleotide identity to DWV-A and has been shown to replicate in *V. destructor* and honey bees (Ongus et al. 2004; Zioni et al. 2011). Recently, Mordecai et al. (2016) have reported the DWV-C, which is a third established variant that has been reported to contribute to winter colony losses (Kevill et al. 2017). It also suggested that the DWV type C has not recently emerged, but also is an established DWV variant. However, the virus has a high ability to mutate and recombine, which hampers the analysis of the sequences.

Several previous studies have established that genomic variations in DWV can affect tropism, pathogenicity, and epidemiology (Gisder et al. 2018; Möckel et al. 2011). This implies that genetic analysis of DWV in honey bee colonies may allow the early identification of colonies at risk of damaging infection and timely implementation of preventive measures. Data on the prevalence of DWV variants in Spain is lacking, although government data suggest the presence of the virus in 83% of colonies and 99% of apiaries (Ministerio de agricultura y pesca 2017).

In the present study, we identified two DWV variants in southern Spain and examined whether genomic differences between them may influence their virulence. DWV load and *V. destructor* infestation were monitored in 10 colonies of an experimental apiary in Andalusia, Spain, over a 21-month period. Of the ten colonies evaluated, two of them were selected based on the following criteria: health status, viral load, and *V. destructor* infestation. One DWV-positive sample from each selected colony was sequenced, and the results were compared with complete DWV genome sequences from the GenBank. Additionally, a follow-up study was based on RT-qPCR of the three DWV master variants (ABC assay) (Kevill et al. 2017). Therefore, the main objective of this study was to determine if the virus sequence was related to the virus virulence. On the remaining colonies, ABC assay (Kevill et al. 2017) was also applied in order to evaluate the distribution of the DWV master variants on the apiary. A secondary objective was to determine what variants of the DWV are present in the South of Spain, which is one of the most important Autonomous Communities regarding beekeeping.

## 2. MATERIAL AND METHODS

### 2.1. Bee sampling and colony health determination

Bees were collected from 10 colonies of an experimental apiary at the Reference Centre for Beekeeping, University of Cordoba, Spain. All colonies were similarly managed. These colonies were studied from March 2015 to January 2017, except for July and August 2015, when sampling was not possible. Colonies were treated against *V. destructor* using Apitraz® in March and September. Samples of adult bees were taken from the hive entrance of each colony, frozen at – 80 °C until analysis, and then analyzed for DWV load

and *V. destructor* infestation level. A total of 149 samples were collected.

Every month, the beekeeping technician inspected all colonies and determined the number of bee, brood, pollen, and honey combs, as well as the presence of DWV symptoms. Colonies with signs of poor population (low activity in the entrance of the colony) or fewer than five bee and brood combs were categorized as having a "poor population." Otherwise, colonies were categorized as having an "adequate population" if they showed high activity level in the entrance of the colony and more than 6–7 frames covered with bees and 2–3 frames covered with capped brood, considering the beekeeping managing and the time of the year. Health-related events were also recorded in the colonies, such as viral symptoms (deformity in wings and nervous symptoms), symptoms of bacterial disease, and mortality.

### 2.2. *V. destructor* load determination

*V. destructor* load was quantified in all colonies throughout the study except for July and August 2015. Mite presence was assessed at each monthly sampling. Mite load was quantified using the soapy water method described in "Standard methods for varroa research" in the COLOSS BEEBOOK (Dietemann et al. 2012).

### 2.3. DWV load determination

DWV load was determined in samples by homogenizing 10 whole bees with mortar and pestle in 5 ml of phosphate-buffered saline (PBS). This amount of starting material should allow detection of DWV if present in more than 25% of bees with a detection probability of 99% at the colony level (Pirk et al. 2013). RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and stored at − 80 °C. This RNA served as template in one-step real-time reverse transcription polymerase chain reaction based on SYBR Green detection as described (Kukielka et al. 2008).

Two colonies were selected based on health status, *V. destructor* infestation level, and viral load, which was classified into the four infection categories defined by Amiri et al. (2015). Health status was defined based on the population size, as assessed from the number of bee/honey/pollen/brood combs, on the presence of DWV symptoms (deformed wings, shortened and rounded abdomens, paralysis), and mortality. Colony H (healthy colony) showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as described below. Colony W (weak colony) showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis.

### 2.4. Whole transcriptome amplification

Total RNA was extracted from the colony H and colony W samples mentioned above and was selected to perform the phylogenetic analysis after amplification using TransPlex Whole Transcriptome Amplification (Sigma-Aldrich). This kit provides a rapid method for preparing amplified cDNA from total RNA for downstream RNASeq applications. It employs a single primer isothermal amplification (SPIA) method to amplify total RNA into double-stranded cDNA and depletes rRNA without preselecting mRNA. In brief, sample homogenate (600 μl) was centrifuged for 10 min at 1792*g*, the supernatant was passed through at 0.45-μm membrane filter, and RNA was isolated using the column-based Nucleospin II Virus® kit (Macherey-Nagel) following the manufacturer's instructions. Total RNA was quantified using the Nanodrop system (Thermo Fisher Scientific, Wilmington, USA) and amplified as cDNA using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, San Luis, USA) according to the manufacturer's instructions, except that each reaction contained 3 μl of template RNA and the amplification involved 30 cycles. Each sample was split into three and processed in parallel. Amplified cDNA was purified using the High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's protocol. Concentration of amplified cDNA was measured

using the Nanodrop (128–297 ng/μl), and the ratio of absorbance at 260/280 and 260/230 nm (Table I). A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA; expected 260/230 values are commonly in the range of 2.0–2.2. Therefore, the obtained values indicated reasonably pure DNA (Wilfinger et al. 1997).

### 2.5. PCR amplification and DNA sequencing

The DWV genomes in the samples from colony H and colony W were sequenced nearly completely using the primer walking approach with 48 PCR primer pairs (Table II), which were designed based on the genomes of DWV (NC_004830.2) and VDV-1 (AY251269.2). PCR was performed using the high-fidelity PrimeSTAR® HS DNA Polymerase (Takara, Saint-Germain-en-Laye, France) in reactions (25 μl) containing 15 μl of 5x PrimeSTAR Buffer, 1 μl of forward primer, 1 μl of reverse primer, 2 μl of cDNA template, and 6 μl of RNAse- and DNase-free water. Reactions were subjected to 35 cycles of denaturation at 94 °C for 2 min, annealing at 62 °C for 30 s, and extension at 70 °C for 5 min in a T3000 thermocycler (Biometra, Göttingen, Germany).

The amplified PCR products were analyzed using 1% agarose gel electrophoresis in 45 mM Tris borate (pH 8.0) and 2.5 mM EDTA (0.53 TBE) containing 0.5 mg/ml ethidium bromide; DNA products were visualized by transillumination with a long-wave UV light box. PCR products were purified using a PCR Purification Kit (Qiagen, Germantown, USA) and >700 bp (excluding primers) were sequenced using the Sanger method on an ABI Prism 3730 (Applied Biosystems, Foster City, CA, USA). The sequencing primers were the same as those used for amplification.

**Table I.** Amounts and purity assessment of cDNA after whole transcriptome amplification

| Sample | Amount (ng/μl) | 280/260 ratio | 230/260 ratio |
|---|---|---|---|
| Colony H-1 | 170 | 1.74 | 2.15 |
| Colony H-2 | 159.4 | 1.81 | 1.9 |
| Colony H-3 | 177.5 | 1.86 | 2.15 |
| Colony W-1 | 214.8 | 1.89 | 2.26 |
| Colony W-2 | 202.3 | 1.80 | 1.58 |
| Colony W-3 | 297.1 | 1.84 | 2.10 |

Colony H and colony W sequences were edited and assembled into nearly complete DWV genomes using MEGA 6 software (Tamura et al. 2013). These sequences were aligned with published sequences using ClustalW. One alignment contained 16 complete genomes of DWV, KV, and VDV (Supplementary data 2), together with the sequences from colony H and colony W. Another alignment was based on the variable RNA-dependent RNA polymerase (RdRp) region in 39 DWV genomes, including the sequences from colony H and colony W. Alignments were edited by hand where necessary based on conserved protein domains as a guide. The two final alignments were considered adequate because the first was associated with an average amino acid p-distance (1—amino acid identity) of 0.074 and the second with an average p-distance of 0.069. These values are within the acceptance threshold of <0.8 (Thompson et al. 1999; Ogden and Rosenberg 2006). From these alignments, phylogenetic trees were constructed using the maximum likelihood method and SPR algorithm and bootstrap testing of 2000 replicates.

The genomes sequenced from colony H and colony W were analyzed for recombination using the Recombination Detection Program 4.1 (Martin et al. 2015), with its algorithms GENECONV (Padidam et al. 1999), BootScan (Martin et al. 2005), MaxChi (Smith 1992), CHIMAERA (Posada and Crandall 2001), SIScan (Gibbs et al. 2000), and 3Seq (Boni et al. 2007). Default parameters and Bonferroni's correction for multiple comparisons were used. $P < 0.05$ was regarded as statistically significant. Only recombination events that more than four algorithms identified as statistically significant were included in further analysis.

**Table II.** Matrix of percent identity from alignment of DWV variants. Colors are used to indicate percent identity: dark blue, > 95%; light blue, 90–95%; orange, 85–90%; yellow, 80–85%

| Sequence | DWV type A | VDV-1 | VDV-1/DWV | DWV Colony H | DWV Colony W |
|---|---|---|---|---|---|
| DWV type A | 100% | 84% | 92.2% | 97.19% | 97.19% |
| VDV-1 | 84% | 100% | 90.2% | 97% | 83.85% |
| VDV-1/DWV | 92.2% | 90.2% | 100% | 88.5% | 91.79% |
| DWV Colony H | 85.3% | 97% | 88.5% | 100% | 85.1% |
| DWV Colony W | 97.19% | 83.85% | 91.79% | 85.1% | 100% |

### 2.6. RT-qPCR ABC assay

For further investigation to the DWV strain distribution in colony H and colony W, as well as within the remaining colonies of the study, ABC assay (Kevill et al. 2017) was performed. In the case of colony H and colony W, all samples collected after the sampling used for sequencing were evaluated using ABC assay. In the remaining colonies, three samples from each colony were analyzed, according to the following criteria: if the colony died during the study (called Dx), samples from the three last samplings before collapsing were selected. If the colony did not die during the study (called Sx), samples from the last three samplings were selected.

One-step, real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), SYBR Green detection, cycling protocols, and primers for DWV-A, DWV-B, and DWV-C previously published (Kevill et al. 2017). Cycling protocol was slightly amended: RT step occurred at 45 °C for 15 min and annealing at 61 °C for 15 s.

Load of positive samples was determined by absolute quantification based on a standard curve constructed using serial 10-fold dilutions of known amounts of PGemT® TA plasmid (Promega, Madison, USA) containing the target genes (RdRp region) of DWV master variant types A, B, and C. Standard curves were fitted with lines showing correlation coefficients of 0.99 (data not shown).

## 3. RESULTS

### 3.1. DWV and *V. destructor* loads in selected samples

Colony H showed no DWV symptoms and survived until the end of the study. A sample from the colony in September 2015 was used for phylogenetic analysis as described above. This sample showed a DWV load of $1.70 \times 10^9$ GEC and *V. destructor* infestation level of 10.68%.

Colony W showed deformed wings and mortality during the study and collapse in November 2016. A sample collected in October 2015 was used for phylogenetic analysis. This sample showed DWV load of $6.70 \times 10^7$ GEC and *V. destructor* infestation of 1.45%.

### 3.2. Colony health during the study

Two DWV genomes from colonies in southern Spain were sequenced for this study. Both genomes were 9031 nt long, excluding 3′ polyadenylated tails (GenBank accession MK262742 and MK262743). One genome came from a colony with good health status, reflected in high population and adequate numbers of bee/honey/

pollen/brood combs. This colony H showed high DWV load in 10 of 21 monthly samplings. *V. destructor* infestation rate was higher at the beginning and end of the study (Figure 1). Despite high DWV and mite levels, the beekeeping technician reported the population to be adequate and stable throughout the study period. Anti-mite treatments successfully decreased mite levels and DWV load. Pearson's correlation analysis showed a positive correlation between the two pathogens ($r = 0.591$, $P = 0.015$). No DWV symptoms were detected in this colony throughout the study.

The second genome came from a colony (colony W) that presented deformed wings, mortality, and smaller population than the other colonies in the apiary. In March 2015, near the beginning of the study, the colony showed high DWV load, which decreased slightly over time (Figure 2). *V. destructor* was present from the beginning of the study but decreased after anti-mite treatment. Toward the end of the study, viral load and *V. destructor* infestation rate increased. This colony died in November 2016, 2 months before the end of the study. Before death, the colony showed high DWV load ($2.4 \times 10^7$ GECs), small population, and the DWV symptom of deformed wings.

In both colonies, *V. destructor* infestation rate and, to a lesser extent, DWV load varied seasonally, with pathogen levels highest during the summer and at the beginning of the fall. Both colonies showed high levels of both pathogens but responded differently to them.

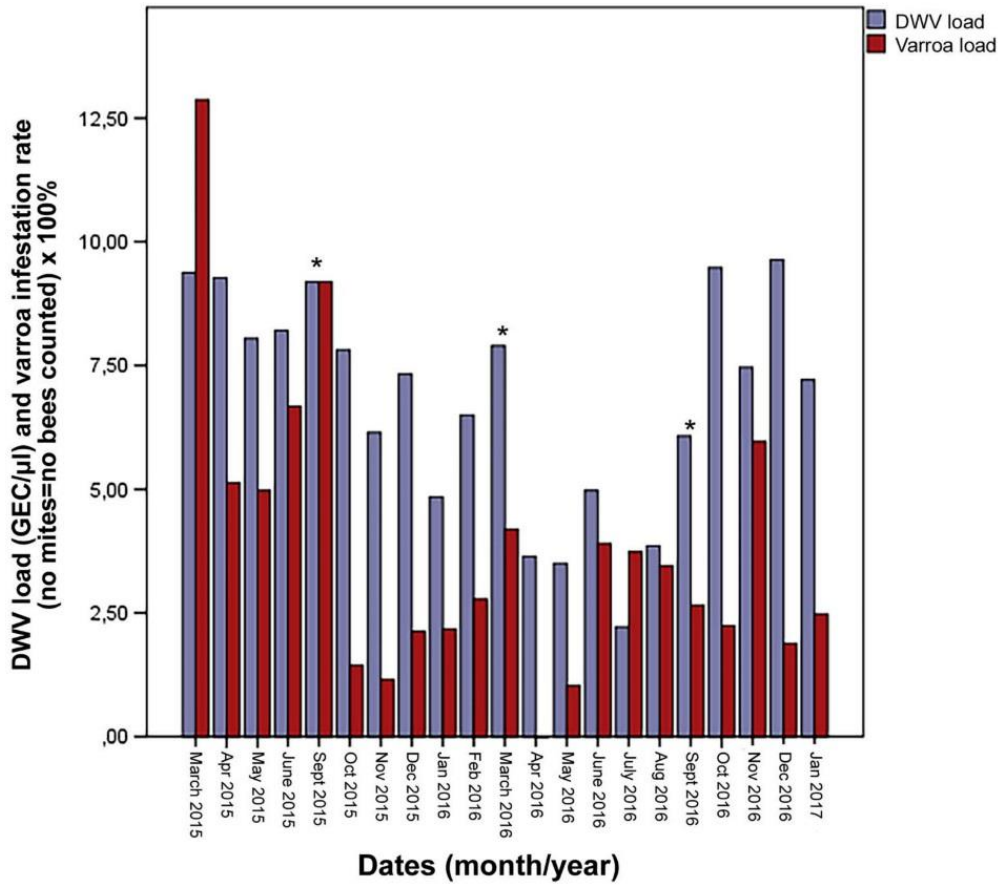


**Figure 1** DWV load and *V. destructor* infestation rate in colony H during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

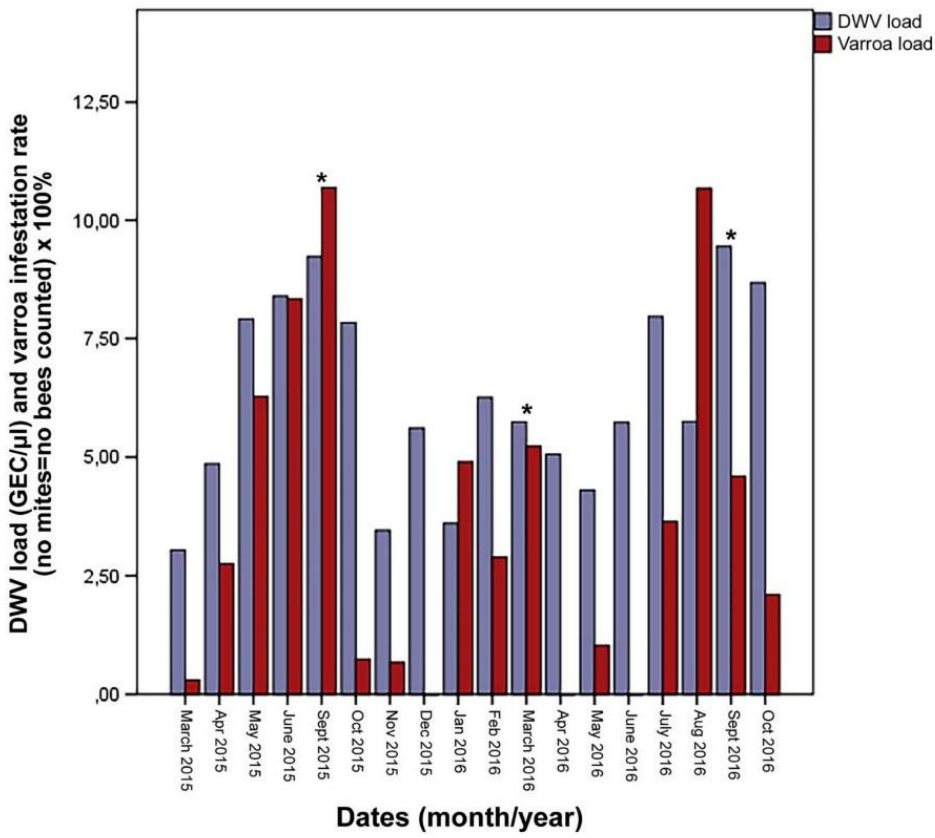


**Figure 2** DWV load and *V. destructor* infestation rate in colony W during the study period. The asterisks mark months when anti-*V. destructor* treatment was applied.

### 3.3. Phylogenetic analysis based on complete DWV genome sequences

Specific primer pairs were applied to both DWV-positive colonies in order to sequence the complete DWV genome (Supplementary data 1). The complete DWV genome sequences from colony H and colony W were aligned with the 16 complete genomes of DWV or VDV-1 available in GenBank (Supplementary data 2). The alignment was 9045 nt long, corresponding to genome sequences determined here (9031 nt) as well as some gaps. The sequences determined here covered 88.5% of the DWV reference genome (NC_004830.2). Alignment of both sequences showed 7088 of 9031 nt (78.5%) to be identical. The nucleotides showed low divergence among the DWV reference genomes used for the alignment.

Two thousand one hundred seventeen variable nucleotide positions were identified across the entire genome. Among segregating sites, the average pairwise nucleotide diversity between sequences was $\pi = 0.073$, and the Tajima $D$ test statistics rejected the neutrality hypothesis ($D = 0.146$). The colony H genome exhibited 85.3% similarity to the DWV-A reference genome (NC_004830.2), 97% similarity to the VDV-1 reference genome (AY251269.2), and 88.5% similarity to the recombinant VDV-1/DWV genome (KX373900.1). The corresponding similarity percentages for the colony W genome were 89% similarity to the DWV type A, 77.1% to the VDV-1, and 84% to the VDV-1/DWV recombinant.

Evolutionary relationships among DWV genomes were inferred using maximum likelihood based on the general time reversible model (Figure 3) (Kumar 2000). The analysis

involved 18 nucleotide sequences, including 1st, 2nd, 3rd, and noncoding codon positions. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. In the end, 9031 positions were analyzed. The bootstrap consensus tree inferred from 2000 replicates (Felsenstein 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches were collapsed if the corresponding partitions occurred in fewer than 50% of bootstrap replicates. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using maximum composite likelihood and then selecting the topology with better log-likelihood value. Differences in rate of evolution among different sites were modeled using a discrete gamma distribution (2 categories, +G, parameter = 0.2136).

Phylogenetic analysis showed that the colony H genome clustered with genomes identified from honey bee colonies without DWV symptoms. These other genomes included one from an experimental apiary in Belgium, the VDV-1 reference genome, and two recombinant VDV-1/DWV genomes from the UK. In contrast, the colony W genome clustered with genomes from colonies with DWV symptoms, mainly deformed wings (Supplementary data 2). The most closely related sequence was from an Austrian colony with losses.

### 3.4. Recombination breakpoints

We examined recombination events in the entire dataset of complete genomes, without any assumption of putative parental sequences. A total of 17 recombination events were detected (Supplementary data 3). This analysis suggested that the colony H genome showed three recombination events. The first recombination event (between VDV-1/DWV-B and DWV-A) was between a genome from Belgium (JX783225.1), which came from a colony that lacked DWV-specific symptoms but showed


**Figure 3** Maximum likelihood phylogenetic analysis of complete DWV genomes. A total of 16 complete DWV, VDV-1, and KV genome sequences were obtained from the GenBank database and aligned with the two nearly complete genomes reported here. Red circles indicate previously published sequences from colonies with symptoms (deformed wings, mortality, low population); green circles indicate previously published sequences from colonies without any symptoms; purple circles mark the two sequences obtained in the present study.

relatively short lifespan, and the DWV reference genome (NC_004830.2), which came from a colony that had deformed wings. It was located at 1045–1685 nt, encoding the VP2 and VP3. The second recombination (VDV-1/DWV-B and DWV-A) was located at 4163–4703 nt, encoding the VP3 and the helicase. The third recombination event (between DWV-A and DWV-A) was located at 8895–9026 nt, encoding the N-terminal region.

The analysis suggested that the colony W genome showed a recombination event between a genome from INRA in Avignon, France (KX373899.1), which came from a colony that showed deformed wings, and a genome from South Korea (JX878304.1), which came from a colony that lacked DWV-specific symptoms.

### 3.5. Phylogenetic analysis based on RdRp regions of the DWV genome

In order to explore phylogenetic diversity based on the variable RdRp region (nucleotide position 9265–9594 in the DWV reference complete genome), we performed a second phylogenetic tree. Analysis based on 37 DWV RdRp sequences in Genbank showed a cluster including DWV and KV genomes and another including VDV-1 genomes. The colony W genome fell within the DWV-KV cluster, while the colony H genome fell within the VDV-1 cluster (Figure 4).

### 3.6. Detection of DWV master variants using the ABC assay

In colony H, ABC assay revealed that DWV-B was the most prevalent variant, although DWV-A was also present, but to a lesser extent. DWV-B load was higher than DWV-A load in this colony. In colony W, DWV-A was detected in all the samples; meanwhile, DWV-B was detected on a smaller number of samples and at lower load (Supplementary data 4).

In the remaining colonies, ABC assay was applied to the 3 months before collapsing (if the colony died) or to the 3 months before the end of the study (if the colony did not died). Five of the eight remaining colonies died before the end of the study period (Supplementary data 5). RT-qPCR results (ABC assay) showed that DWV-A and DWV-B variants were detected in honey bee samples selected from the other eight colonies; meanwhile, DWV-C was not detected in any sample. DWV type A was detected in the majority of samples tested, being the dominant variant in colonies that collapsed. On the other hand, DWV type B was also quite prevalent and achieved higher load in surviving colonies.

## 4. DISCUSSION

Positive-strand ssRNA viruses are associated with honey bee colony losses; in particular, the combination of DWV and *V. destructor* has been related to strong virulence and severely colony mortality (Dainat et al. 2012). Field evidence have demonstrated that the presence of DWV and *V. destructor* is an important factor contributing to the current colony losses, but how both pathogens interact so effectively to trigger colony losses requires further study. Phylogenetics suggests that the mite may be driving changes in the DWV genome (Mordecai et al. 2016a; Mordecai et al. 2016b; Ryabov et al. 2014), but it is unclear whether such genetic changes affect viral virulence and therefore risk of colony loss (McMahon et al. 2016; Natsopoulou et al. 2017). The present study assessed whether nucleotide variations in the DWV genome can influence virulence. Our results suggest that different DWV variants at the same load can trigger different effects on colonies in the same apiary that are exposed to the same conditions and show similar *V. destructor* infestation. These findings suggest that nucleotide differences in the DWV strain can make the difference between colony health and collapse, implying that DWV genotyping of colonies may facilitate early identification of colonies at greater risk of collapse. At the same time, our study provides the first nearly complete sequences of DWV isolates from southern Spain, where the beekeeping sector is more professional than in other parts of the country.

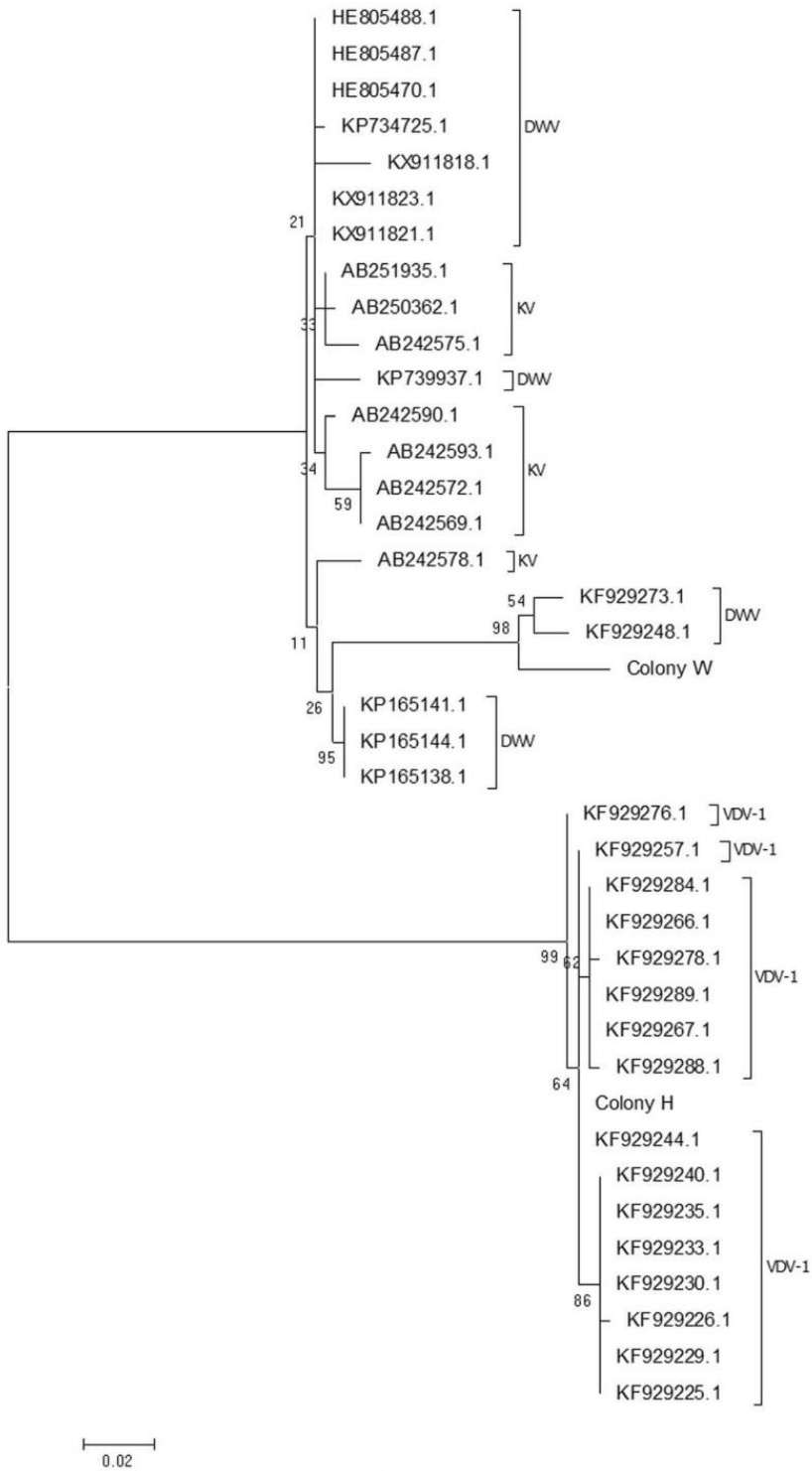


**Figure 4** Maximum likelihood phylogenetic analysis based on the variable RdRp region in the DWV genome. A total of 35 RdRp sequences from DWV, VDV-1, and KV in GenBank were compared with the two RdRp sequences determined here.

We were able to sequence nearly the entire DWV genomes from the two colonies in our study, including the internal ribosome entry site, the variable RdRp sequence, and the sequences encoding the L protein, structural proteins, and helicase. We were unable to sequence two open reading frames of 949 and 161 nt, flanked respectively by the 5′ or 3′ non-translated region, or the poly(A) tail. The colony H genome (accession number MK262742), from a healthy colony, showed recombination at three breakpoints between the DWV reference genome from Italy (Lanzi et al. 2006) and a DWV sequence from Belgium (Benaets et al. 2017). The colony H genome clustered with sequences that mainly came from healthy colonies, including the VDV-1 reference genome (97% similarity) and recombinant VDV-1/DWV genomes from the UK (Moore et al. 2011). Despite the fact that the colony H genome mainly clustered with VDV-1/DWV-B genomes, it was a recombinant between VDV-1/DWV-B and DWV-A. Therefore, both DWV-A and DWV-B wild-type genomes must have been present simultaneously. For this to be the case, the DWV-A genome has had to diverge significantly enough to mainly cluster with VDV-1/DWV-B and not with the other DWV-A/DWV-B recombinants. It showed three recombination events throughout its genome. The first recombination event was located in a similar region of the genome as reported by Mordecai et al. (2016a), between the Lp and the VP2. However, the second recombination event was located in the VP3 and the helicase region. Therefore, it is, to our knowledge, the first report of a recombination between DWV-B and DWV-A in this region of the genome. The colony W genome (accession number MK262743), from a weak colony with DWV symptoms, showed one recombination event between the same sequence from Belgium and an isolate from South Korea (Reddy et al. 2013). The colony W genome clustered with sequences from colonies with symptoms and mortality. It also clustered with the KV reference genome. Similar clustering results for the colony H and colony W isolates were obtained in phylogenetics analyses based only on the variable RdRp sequence.

Despite not all the nucleotides of the DWV sequences from the Spanish apiary were sequenced, our primer walking approach covered part of the IRES, L protein, and the structural proteins, as well as the helicase and the RdRp, in order to study recombination events in these regions of the viral genome. These results show that substantial genetic variation can be found in DWV strains affecting the same apiary. Indeed, the isolates from colony H and colony W showed only 85.5% similarity. This variation may help explain differences in viral virulence (Mordecai et al. 2016a; Mordecai et al. 2016b) and therefore colony outcomes such as in the present study. Our results are consistent with previous reports that VDV-1, DWV-B, and their recombinants are less virulent than DWV-A and KV (Mordecai et al. 2016a). Our findings justify further work into the potential association between DWV nucleotide sequence and virulence.

While differences in DWV virulence on their own may explain why colony H survived with good health despite high viral load and *V. destructor* infestation rate, another potential factor is the so-called superinfection exclusion (Folimonova 2012). According to this concept, an established virus infection can interfere with secondary infection by the same or a closely related virus. This exclusion may benefit the established virus because it reduces competition for resources and stabilizes its genome by reducing the risk of recombination with other viral genomes in the same cell. This exclusion may help explain how the mite can decrease DWV diversity by making certain DWV variants dominate over others (Mordecai et al. 2016a). Superinfection exclusion may protect the honey bee colony if an established infection with a weakly virulent DWV strain (e.g., transmitted by the mite) prevents secondary infection with virulent DWV-A. Under the assumption that DWV-B can be regarded as weakly virulent, and taking into account that colony H genome was a DWV-B/DWV-A recombinant, our results provide the first supporting evidence of superinfection exclusion at the colony level by a recombinant DWV-A/DWV-B (more similar to DWV-B). One of the issues that emerges from this finding is if the region of the genome where recombination occurs

plays a key role in determining DWV virulence, since the recombinant DWV from colony H protected the colony. This should be also carefully confirmed in light of reports that DWV-B may be correlated with losses in worker number over the overwintering period, as it was suggested by a recent study carried out by Natsopoulou et al. (2017). McMahon et al. (2016) also probed, under laboratory conditions, that DWV-B could be more virulent than the DWV-A variant. In this experiment, the authors observed higher mortality in the DWV-B-infected group, compared to the mock-infected control group. However, these experiments need to be replicated in honey bees from different geographical locations, at different viral loads, and under field conditions to test this conclusion. All of these variables make it difficult to understand the current role of variant DWV-B.

When exploring RdRp region by constructing a phylogenetic tree, sequence from colony H clustered with VDV-1 sequences and sequence from colony W clustered with DWV and KV sequences. These results confirmed the similarity between the sequences from this study and the stipulated DWV variants.

ABC assay on colonies H and W revealed that the distribution of DWV-A and DWV-B variants was relatively constant over the study period. DWV-A was detected in both colonies, but high DWV-B loads may have protected colony H from DWV-A, since DWV-A was present at lower levels and this colony survived the study period and did not show any symptoms. On the contrary, colony W showed higher DWV-A loads and lower DWV-B loads, which may have contributed to its collapse. DWV-A titles over $10^7$ GEC were detected in colony W the months before collapsing; meanwhile, DWV-B was not detected in some samplings or it was present at low levels. This result may be explained by the fact that DWV-B was not in sufficiently high levels to protect the colony from the virulent DWV-A.

Additionally, ABC assay on colonies that died revealed that DWV-A was the dominant variant in the 3 months before collapsing. By contrast, colonies that survived showed lower DWV-A load and higher DWV-B load, which could have had a protective effect on those colonies. These findings are consistent with those of other studies (Kevill et al. 2017; Martin et al. 2012; Mordecai et al. 2016a; Schroeder and Martin 2012) and support the hypothesis that DWV-A can be more virulent but high DWV-B titles may have a protective effect on colonies. However, these data must be interpreted with caution because of the low sample size, and further study of the potential protective effect of DWV-B should be done. Surprisingly, DWV-C was not detected in any sample, which may reveal that this variant is not established in southern Spain yet. Therefore, DWV-C implications on colony health could not be assessed.

Our study indicates the presence of at least two DWV strains in southern Spain: VDV-1/DWV-B recombinant with DWV-A (more similar to DWV-B) (DWV-SpB) and DWV-A (DWV-SpA). These findings extend our limited understanding of the global distribution of DWV variants (Cornman et al. 2013; Kielmanowicz et al. 2015). Further work, which considers these results, should examine the prevalence of variants in Spain and more broadly in Europe, since Europe has been reported to be a critical source for the global spread of the DWV variants.

Recombination breakpoints were identified in the colony H genome at 1095–1685 nt in the regions encoding VP2 and VP1, as well as at 4163–4703 nt in the region encoding the VP3 and the helicase. These two breakpoints were derived from the DWV-A reference sequence. A third breakpoint was identified at 8895–9026 nt in the N-terminal region, derived from the DWV sequence from Belgium. The colony W genome showed a recombination breakpoint at 3828–4408 nt overlapping with the region encoding the helicase and derived from a South Korean isolate showing deformed wing symptoms. These results clearly show that honey bee colonies can be infected by a mixture of DWV and its recombinants, which may result from multiple modes of virus transmission.

In this study, we report the nearly complete genome sequences of two DWV isolates from an apiary in southern Spain and show that they correspond to two DWV variants. We show evidence of genetic diversity in DWV populations and for an association between genomic sequence and viral virulence. Genetic changes in DWV may help it adapt to its host and its vector (*V. destructor*). In

conclussion, our findings add evidence to a growing body of literature on the genetic study of DWV and emphasize the importance of understanding pathogen genetic diversity when investigating causes of honey bee losses.

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## AUTHOR CONTRIBUTIONS

SB, MVR, and JSV designed experiments, FP helped in the interpretation of the data, SB and FMR performed experiments and analysis, and SB wrote the paper. All authors read and approved the manuscript.

**Des variations de séquence nucléotidique peuvent être associées à la virulence du virus de l'aile déformée**

**virus de l'aile déformée / *V. destructor* / phylogénie / abeille mellifère**

**Variationen der Nukleotidsequenz sind möglicherweise mit der Virulenz des Flügeldeformationsvirus verknüpft**

**flügeldeformationsvirus / *V. destructor* / virusvirulenz / phylogenie / honigbiene**

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# Chapter 2

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# Resumen

Las abejas poseen distintos mecanismos de defensa ante los patógenos. A pesar de no tener un sistema inmunitario de tipo innato, cuentan con un sistema inmunitario individual, además de contar con un sistema inmunitario de tipo colectivo. Si nos centramos es el sistema inmunitario individual, la primera barrera defensiva que presentan las abejas es el tegumento, seguido por la respuesta de tipo celular y la humoral. Ésta última engloba distintas rutas inmunitarias, que son activadas en presencia de diferentes agentes y que dan lugar a la expresión de genes que desencadenan respuestas tales como la endocitosis o la melanización.

En este capítulo se describe el estudio de tres de las principales vías inmunitarias (Imd, Toll y JAK/STAT) de las abejas mediante el análisis de expresión de cuatro genes *(relish, defensin, domless* y *dorsal*), con el objetivo de desarrollar marcadores del sistema inmunitario que permitan la detección temprana de problemas en la colmena. Para ello, se evaluó la expresión de estos cuatro genes de manera mensual durante un periodo de un año en siete colmenas de *Apis mellifera* localizadas en un colmenar experimental de Córdoba, así como la carga de cuatro virus de las abejas (DWV, BQCV, SBV e IAPV) y la infestación por el ácaro *Varroa destructor*. Se evaluó, además, la fortaleza y capacidad de supervivencia de las colmenas.

La expresión del gen *relish* estuvo correlacionada positivamente con los niveles de DWV y varroa, mientras que *defensin* presentó regulación negativa en presencia de estos dos patógenos, mostrando niveles de expresión más bajos en aquellas colmenas que no sobrevivieron al estudio con respecto a las que sí. Por otro lado, la expresión del gen *dorsal* mostró correlación negativa con la carga de los virus DWV y BQCV, lo que apoya teorías previas de que la ruta Toll está involucrada en la respuesta inmunitaria frente a los virus. Además, se detectaron tendencias temporales asociadas a las distintas estaciones del año para algunos de los genes

evaluados. Por ejemplo, la expresión de *relish* se encontró acentuada en los meses de verano, al igual que en el caso de *domeless. Defensin,* sin embargo, presentó niveles de expresión más elevados durante el invierno.

Estos resultados han permitido el desarrollo de potenciales marcadores del sistema inmunitario de las abejas, basados en la evaluación periódica de los genes *relish* y *defensin.* La interpretación adecuada de estos genes podría aportar valiosa información acerca del estado de salud de las colmenas, de tal modo que niveles elevados de *relish* indicarían altos niveles de infección por DWV-varroa, mientras que la regulación negativa de *defensin* indicaría que la colmena podría tener dificultades en responder a la infección.

# Abstract

Honey bee population decline threatens the beekeeping sector, agriculture and global biodiversity. Early detection of colony mortality may facilitate rapid interventions to contain and prevent mortality spread. Among others, deformed wing virus (DWV) is capable of inducing colony losses, especially when combined with *Varroa destructor* mite. Since the bee immune system plays a crucial role in ensuring that bees are able to face these pathogens, we explored whether expression of immune genes could serve as biomarkers of colony health.

Herein, we describe a preliminary immunological marker composed of two immune genes (*relish* and *defensin*), which provide insight on honey bee antiviral defense mechanism. Of the tested genes, relish expression correlated with the presence of DWV-Varroa complex, while decreased *defensin* expression correlated with poor resistance to this complex.

The monitoring of these genes may help us to better understand the complex physiology of honey bees´s immune system and to develop new approaches for managing the health impacts of DWV infection and varroa infestation in the field.

# 2.1 Introduction

The western honey bee *Apis mellifera* plays a critical role in pollination of important crops, but high annual losses in the US (Brutscher et al., 2016; Kulhanek et al., 2017) and over-wintering colony losses in Europe have had significant negative consequences on the environment and economy (Hung, Kingston, Albrecht, Holway, & Kohn, 2018). Both of these depopulation processes are poorly understood and are thought to be caused by multiple factors, such as high levels of pathogens, parasites, environmental pollutants, nutritional stress, inadequate beekeeping management and climate change (Genersch, 2010; Ratnieks & Carreck, 2010). Generally speaking, pesticides and pathogens have been reported to be important factors contributing colony losses. The available evidence seems to suggest that collapsing and weak colonies have a greater prevalence of pathogens compared to healthy colonies (vanEngelsdorp, Evans, et al., 2009). On the other hand, laboratory studies have demonstrated that exposure to sub lethal doses of pesticides can negatively affect honey bee behaviour (Eiri & Nieh, 2012; El Hassani et al., 2008), foraging (Henry et al., 2012) and longevity (Wu, Anelli, & Sheppard, 2011). However, only neonicotinoid exposure has been reported to act synergistically with pathogens, by reducing immune defences and promoting the replication of the DWV in honey bees (Di Prisco et al., 2013).

Several pathogens and parasites have been associated with honey bee colony losses, especially the *Varroa destructor* mite and deformed wing virus (DWV), which have been described as predictive markers of winter losses (Dainat et al., 2012b; F. Nazzi & F. Pennacchio, 2018). These two agents are interrelated: *Varroa destructor* harms colonies directly by feeding on honey bee haemolymph, and it harms colonies indirectly by facilitating the transmission of DWV and other viruses. In addition to viral transmission, immunosuppression of the developing honeybee by *Varroa destructor* has been suggested to explain the synergetic relationship between DWV and the mite. However, a recent study carried out by Kuster et al. (2014) (Kuster,

Boncristiani, & Rueppell, 2014) revealed that mite feeding activity itself and not immunosuppression may be the cause of this synergy. Several studies have associated this mite-DWV interaction to increased risk of winter losses (Kuster et al., 2014) As for DWV, different genetic variants have been described (Berenyi et al., 2007; Lanzi et al., 2006). In fact, the mite may even drive selection for more pathogenic variants of DWV, increasing the likelihood of colony collapse (Martin et al., 2012; Mordecai, Brettell, et al., 2016; Mordecai, Wilfert, et al., 2016).

These results suggest that assaying levels of *Varroa destructor* or DWV in a colony may predict colony death. However, colonies have been shown to survive even in the presence of high DWV load (Locke, Forsgren, & de Miranda, 2014). Therefore, being able to distinguish between a normal situation and a pathogenic one is crucial for establishing a proper colony monitoring. As reported by Nazzi et al. (2018) (Nazzi & Pennacchio, 2018), the molecular analyses have revealed that the immune system of honey bees may be determinant in the modulation of this synergistic association. An immune-suppressive syndrome, characterized by a negative transcriptional regulation of several genes, may drive the conversion from "covert" to "overt" infection. This immune suppression can easily trigger colony mortality (Yang & Cox-Foster, 2005), since the immune system of individual bees plays a key role in colony health status (Boutros, Agaisse, & Perrimon, 2002; Wilson-Rich, Dres, & Starks, 2008) together with colony-level anti-pathogen measures such as social hygiene and other colony-level behaviors (Locke et al., 2014).

Knowledge of honey bee immune mechanisms is mostly resulting via comparison to the better-characterized immune responses in fruit-flies and mosquitoes. General aspects of immunity, including detection of pathogen associated molecular patterns (PAMPs) and production of effector molecules are conserved in mammals, plants, and insects, and both plants and insects employ RNA interference (RNAi) as a major mechanism of antiviral defence (Ding, 2010; Ronald & Beutler, 2010). The individual innate response comprises a humoral and cellular

immune response (Evans et al., 2006; Scott Schneider, DeGrandi-Hoffman, & Smith, 2004). Cellular response consists in phagocytosis, encapsulation and melanization mechanisms (Osta, Christophides, Vlachou, & Kafatos, 2004). Both nodulation and encapsulation are frequently accompanied with melanization, which are catalysed by pro-Phenoloxidase (PO) (Decker & Jaenicke, 2004). The humoral response involves secretion of antimicrobial peptides, melanisation, and the enzymatic degradation of pathogens (Evans, 2004). The innate immune system in honey bees is composed of pattern recognition receptors (PRRs) that interact with pathogen-associated molecular patterns (PAMPs), stimulating different pathways as a function of each type of pathogen. Gram-positive bacteria and/or fungi are thought to stimulate both the Toll pathway, leading to up-regulation of *dorsal,* and the Immune Deficiency (Imd) pathway, leading to up-regulation of *relish* (Galiana-Arnoux, Dostert, Schneemann, Hoffmann, & Imler, 2006). Viruses, for example, trigger mainly the RNA interference pathway (Evans, 2006; Hillyer, 2016), although DWV infection in honey bees also down-regulates *dorsal*, suggesting inhibition of the Toll pathway (Van Rij et al., 2006); in fact, RNAi mediated silencing of this gene was clearly associated with increased viral replication (Nazzi & Pennacchio, 2018).

Thus, there is evidence that the immune system plays a crucial role in ensuring colony survival and that honey bees have innate immune mechanisms to fight against infections that have been related to colony mortality (Boutros et al., 2002). However, although advances in elucidating these immune mechanisms have been reached in last years, it is not fully understood how particular infections trigger complex responses in colonies and how these responses evolve throughout the seasons. Hence, thorough studies of the biological significance of most genes *in vivo* are required.

The present study explored whether expression levels of four immune system genes could serve as biomarkers of elevated risk of colony mortality. This preliminary immune marker was defined in relation to the worst pathogen scenario

that honey bees have to face: the joint action of *Varroa destructor* and DWV, which may be extrapolated to other infectious diseases. In seven honey bee colonies in one apiary in Spain, we examined possible correlation of *Varroa destructor* and DWV load with expression of four *A. mellifera* genes involved in honey bee immunity and colony health status for ten months. Both pathogens have been described as predictive markers of honey bee colony collapse (Dainat et al., 2012b) and their monitoring may help beekeepers to establish preventive measures. However, they do not provide enough information about colony health status, since the colony is able to deal with infectious pathogens on many times if its immune system works properly. Thus, this study describes a preliminary marker based on immune system response, which provides not only information about pathogens affecting the colony, but also of how the colony is facing them. This marker is based on evaluating relish and *defensin* expression through qPCR analysis. *Relish* expression reflects levels of DWV infection and varroa infestation, while *defensin* expression reflects how well the colony can resist these pathogens. To monitor such double immune marker in the most critical moments (winter, extreme temperatures, high *Varroa destructor* infestation, risk management) would help beekeepers to set up preventive measures and to standardize honey bee colony monitoring. However, further studies should be conducted to test the application of this double immune marker under different environmental conditions.

# 2.2. Material and methods

## 2.2.1 Experimental design

An experimental apiary of seven Langstroth hives of *Apis mellifera* was established in the Reference Centre for Beekeeping at the University of Cordoba (Cordoba, Spain). During the period from March 2015 to April 2016, all colonies were sampled monthly except for July and August, when sampling was impossible due to high temperatures in the apiary. At each sampling, a beekeeping technician inspected

colonies; determined numbers of honey bees, brood, pollen and honey combs using the subjective method as described (Delaplane, van der Steen, & Guzman-Novoa, 2013b); and noted the presence of symptoms, mortality and depopulation. Samples of approximately 50 adult bees were carefully taken by hand from the hive entrance or the honey combs of each colony and frozen at -80 °C until analysis. Sampling process was systematically repeated among colonies in order to obtain the most homogenous sample under field conditions

### 2.2.2 Quantification of *Varroa destructor* load

*Varroa destructor* load was quantified monthly in all colonies throughout the study except for July and August 2015. Mite presence was assessed at each monthly sampling. Mite load was quantified using the soapy water method described in "Standard methods for varroa research" in the COLOSS BEEBOOK (Dietemann et al., 2012). Briefly, 300 adult bees were collected from the colony from the sides of the unsealed brood combs, shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. The percentage of mites was calculated as follows:

$$\% \text{ infestation} = (\text{no. mites} / \text{no. bees counted}) \times 100\%$$

After sampling and inspection in September 2015 and March 2016, colonies were treated with oxalic acid against the mite.

### 2.2.3 RNA extraction

Ten intact bees were homogenized in 5 ml phosphate-buffered saline (PBS, pH 7.2) with mortar and pestle, and total RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel) according to the manufacturer's instructions. Total RNA was suspended in RNase/DNase-free water and stored at −80◦C (RNA).

### 2.2.4 Virus testing

RNA samples were assayed to determine load of four bee viruses: DWV, black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV) and sacbrood bee virus (SBV). One-step, real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), SYBR Green detection and primers and cycling protocols previously published for DWV (Kukielka, Esperón, et al., 2008), BQCV (Kukielka, Esperón, et al., 2008), IAPV (Maori et al., 2009) and SBV (Amiri et al., 2015).

Viral load of positive samples was determined by absolute quantification based on a standard curve constructed using serial 10-fold dilutions of known amounts of PGemT® TA plasmid (Promega) containing fragments of DWV and BQCV. Standard curves were fitted with lines showing correlation coefficients of 0.99 (data not shown). Viral loads were expressed in absolute terms in terms of RNA equivalents per microliter (RNA equivalents/µl), and in relative terms using a 4-point scale (Amiri et al., 2015): free of virus (RNA equivalents /µl=0), low virus load (0<RNA equivalents/µl<$10^3$), medium virus load ($10^3$≤RNA equivalents/µl<$10^7$) and high virus load (RNA equivalents/µl≥$10^7$). This procedure can detect virus that has infected at least 25% of a colony with 95% probability (Pirk et al., 2013).

### 2.2.5 *Nosema cerana* testing

To extract DNA for microsporidia, the protocol of BeeBook was adapted (Fries et al., 2013). Two hundred µl of the homogenates used for virus testing were centrifuged at 16,100g and supernatant was discarded. Pellets were frozen and crushed with sterile tips to disrupt nosema spores. This process was repeated three times before extraction of DNA with DNA Isolation kit (Roche), following manufacturer instructions. DNA was frozen to -20ºC until molecular analysis. One-step real time polymerase chain reactions (qPCR) based on SYBR-Green dye and using

primers and PCR conditions previously described by Forsgren and Fries (2010) (Forsgren & Fries, 2010).

### 2.2.6 Expression of immune system genes

Genes involved in three inducible immune pathways in honey bees were studied: Toll, Janus kinase (JAK/STAT) and Imd (Evans et al., 2006). Expression of the following genes was measured using specific primers: *defensin*-1 (Aronstein, Murray, & Saldivar, 2010), *dorsal* (Evans, 2006), *domeless* (Evans, 2006) and *relish* (Evans, 2006). Total RNA extracts obtained as described above were used to prepare cDNA with the PrimeScript RT Reagent Kit (Clontech, Takara). RNA extract (2 µl) was incubated with 2 µl of PrimeScript Buffer, 0.5 µl of PrimeScript RT enzyme, 0.5 µl of oligo(dT) primer, 0.5 µl of Random 6 and 4.5 µl of RNase/DNase-free water for 15 min at 37 ºC and 5 sec at 85 ºC. The resulting mixtures were diluted 1:10 with molecular biology-grade water for a total of 100 µl cDNA template for quantitative PCR. All samples were analysed in parallel using a SYBR Fast Universal qPCR system (KAPA Biosystem) (Pirk et al., 2013).

Individual reactions contained 5 µl of master mix (buffer and enzyme), 2 µl of cDNA template, 0.5 µl of forward and reverse target primers (5 µmol, l:1), and 2.2 µl of molecular biology-grade water. Reactions were cycled on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the following conditions: 5 min at 95 °C followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and 72º for 7 s, during which fluorescence measurements were taken. A final melt curve analysis was conducted at 95°C for 15 s and 65°C for 15 s. Each target gene was assayed for all samples on a single plate. A sample in which levels of all four viruses were undetectable was analysed to provide an uninfected reference as a default value in the Ct analysis.

Levels of expression of target genes were normalised to that of the β-actin gene. These normalised expression levels were compared among the seven colonies and between colonies positive or negative for each virus.

### 2.2.7 Statistical analyses

Study variables are shown in Table 1. IAPV load and SBV load were not included in the final analysis, since all samples tested negative for these viruses. Differences in all variables were assessed for statistical significance using the non-parametric Mann-Whitney U test, since the data for all variables showed a skewed distribution. Virus load data were $\log_{10}$-transformed before statistical analyses, which were performed using SPSS version 22.0 (IBM, 2013). $P<0.05$ was considered significant.

Colonies were also classified according to their DWV load, i.e. high, medium, low DWV load and free of virus according Amiri et al. (2015), in order to establish differences between immune gene level expressions in these groups.

Using multivariate Spearman correlation analysis, potential correlations among DWV, BQCV and *Varroa destructor* loads were explored across all seven colonies of the apiary over the 10 months of the study. Then, each colony was analyzed separately for these correlations. In the same way, Spearman correlations between pathogens and immune system gene expression were analyzed firstly across all colonies in the apiary and then for each colony. However, statistical analysis focused on individual colonies due to two reasons: 1) only three colonies survived for the entire period of study, which limited the analysis and 2) colonies are super organisms, at least in terms of their basic physiology, therefore individual conditions could be determinant for each colony. Immune system gene expression was also compared among colonies showing undetectable, low, medium or high virus load. Differences in study variables between collapsed and survived colonies were assessed for significance using the Mann-Whitney *U* test.

**Table 1.** Summary of study variables.

| # | Variable | Type | n |
|---|---|---|---|
| **1** | Colony ID | Categorical | 7 |
| **2** | DWV load | Continuous | 57 |
| **3** | BQCV load | Continuous | 57 |
| 4 | *Varroa destructor* infestation rate | Continuous | 57 |
| 5 | *Relish* expression value | Continuous | 57 |
| 6 | *Defensin* expression value | Continuous | 57 |
| 7 | *Domeless* expression value | Continuous | 57 |
| 8 | *Dorsal* expression value | Continuous | 57 |
| 10 | Spring-Summer season | Categorical | 28 |
| 11 | Month | Categorical | 12 |

# 2.3 Results

Four colonies collapsed during the study: colony 2 (May 2016), colony 3 (May 2015), colony 4 (June 2015) and colony 5 (July 2015).

## 2.3.1 Viral load

IAPV and SBV were not detected in any sample. DWV was more prevalent than BQCV throughout the study period. Nearly all samples (97.5%) were positive for DWV, with load ranging from 2.75 x $10^2$ to 2.39 x $10^9$ RNA equivalents/µl. Colony 3 had the highest mean DWV load (2.39 x $10^9$ RNA equivalents/µl), and it rapidly collapsed before the summer. Colony 1 had the second-highest mean DWV load. Both colonies also showed the highest rates of *Varroa destructor* infestation, at 20.43% and 4.42%, respectively. Mean DWV load was 2.04 x $10^5$ RNA equivalents/µl during the winter and 2.69 x $10^6$ RNA equivalents/µl during the warmer months.

BQCV showed a mean prevalence of 89.43% and mean load of 1.66 x $10^3$ RNA equivalents/µl. Colony 4 showed the highest mean BQCV load (4.37 x $10^3$ RNA

equivalents/µl), followed by colony 6 (3.72 x $10^3$ RNA equivalents/µl). Mean BQCV load was 2.99 x $10^2$ during the winter and 2.88 x $10^3$ during the warmer months.

### 2.3.2 *Varroa destructor* infestation

In colonies that survived until the end of the study, the mite was present for at least 9 of the 12 samplings, and infestation rates varied from 0.3 to 28.85% (Figure 1). *Varroa destructor* infestation rates were higher in warmer months (May, June, and September) and lower in autumn and winter months, following acaricide treatment. The rate dropped significantly between September and October following oxalic acid treatment ($p=0.021$, Mann Whitney test). Colony 3 showed *Varroa destructor* infestation rates >10% in March and April 2015, and it collapsed in May 2015. The combination of high mite infestation and high DWV load may be the primary causes of the collapse.

*Varroa destructor* infestation rate correlated with DWV load across all seven colonies over the entire study period ($rs=0.648$, $p<0.001$), as well as specifically in colonies 1 ($rs=0.829$), 4 ($rs=1$) and 7 ($rs=0.648$, all $p<0.05$).

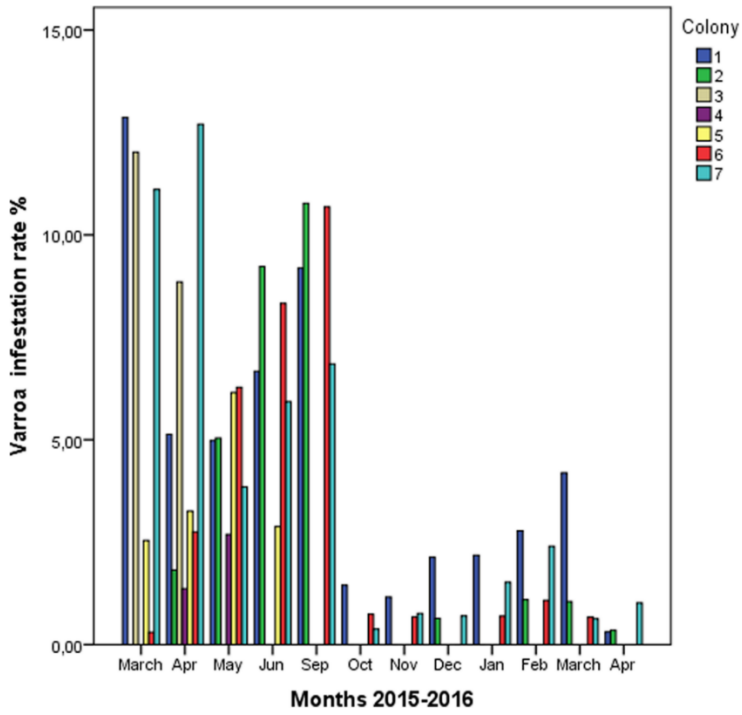


**Figure 1.** Rates of *Varroa destructor* infestation (per 100 adult bees) in each colony based on monthly sampling.

### 2.3.3 *Nosema ceranae* infection

Nosema ceranae was not detected in any sample.

### 2.3.4 Correlations among the immune pathways studied

Comparison of levels of expression of the four immune system genes from three immune response pathways (Figure 2) revealed three positive correlations among the pathways (Table 2). One correlation occurred between *relish* and *defensin* ($r_s$=0.405, p=0.002), reflecting the production of antimicrobial peptides via the Imd pathway. Another correlation occurred between *relish* and *domeless* ($r_s$=0.707, p<0.001), reflecting the fact that the Jak-STAT pathway is activated by viruses and Gram-

negative bacteria, although we did not test bacterial load. A third correlation was observed between *domeless* and *defensin* (rs=0.630, p=0.001).

**Table 2.** Correlations in relative expression levels between pairs of immune system genes, based on the data shown in Figure 2.

| Interaction | Correlation coefficient | p | n |
|---|---|---|---|
| ***Relish-defensin*** | 0.405 | 0.002 | 57 |
| ***Relish-domeless*** | 0.707 | <0.001 | 57 |
| ***Defensin-domeless*** | 0.630 | <0.001 | 57 |
| ***Defensin-dorsal*** | 0.539 | <0.001 | 57 |
| ***Domeless-dorsal*** | 0.470 | <0.001 | 57 |

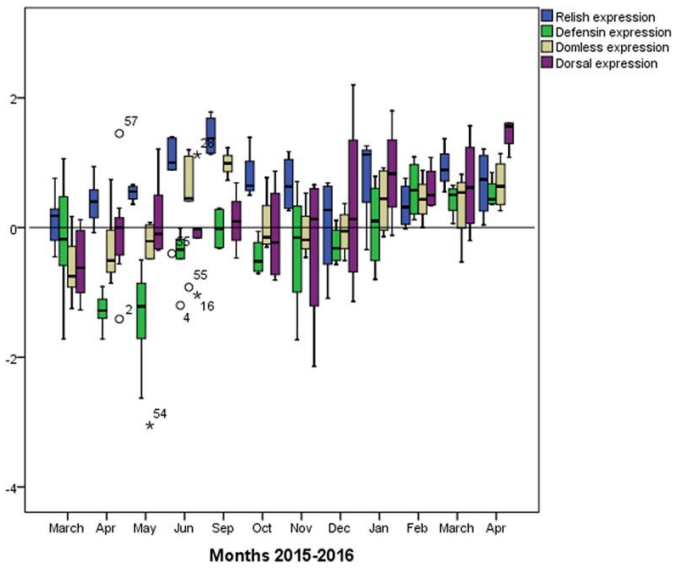


**Figure 2**. Relative expression of immune system genes across all seven colonies in the apiary during the study period. Expression levels are expressed relative to adjusted for the levels observed in uninfected reference samples. The solid horizontal line indicates relative expression equal to that in the uninfected reference.

### 2.3.5 Apiary-level analysis of immune response to DWV infection and *Varroa destructor* infestation

Expression of *defensin* correlated negatively with DWV load (rs=-0.385, p=0.008) and mite load (rs=-0.354, p=0.13). Expression of *domeless* correlated negatively with BQCV load (rs=-0.334, p=0.011). Expression of *dorsal* correlated negatively with BQCV load across the study period (rs=-0.277, p=0.039) and with DWV load during the spring-summer season (rs=-0.509, p=0.003). Conversely, expression of relish correlated positively with DWV load during the spring-summer season (rs=0.403, p=0.042).

In addition to these analyses in which DWV load was treated as a continuous variable, we analysed the load in categorical terms of low or high. Expression of *dorsal* was significantly lower in colonies with high load than in those with low load (Mann-Whitney U test, p=0.013). Conversely, expression of relish was significantly higher in colonies with high DWV load than in those with low load (Mann-Whitney *U* test, p=0.049).

### 2.3.6 Colony-level analysis of immune response to DWV infection and *Varroa destructor* infestation

Significant relationships between immune system gene expression and pathogen load were detected within individual colonies (Table 2). High DWV and mite loads were usually associated with an increase in relish expression (Figure 3) but with a decrease in *dorsal* and *defensin* expression (Figures 4-5). In fact, in colonies 1, 6 and 7, which survived the entire study period, an increase of *dorsal* expression in the winter was accompanied by a decrease of DWV load. However, colonies 2 and 4 showed increased of *dorsal* expression in the month prior to collapse. In colony 2, *relish* expression correlated positively with *domeless* expression (r=0.909, p<0.001) and *dorsal* expression (r=0.783, p=0.003).

**Table 3.** Correlations between pathogen load and immune system gene expression. All correlation analyses were performed using data from the whole period of study, with exception of the DWV-*dorsal* correlation, which was analysed using data from the spring-summer season.

| Interaction | Correlation coefficient | p | n |
|---|---|---|---|
| **DWV-*defensin*** | -0.385 | 0.008 | 57 |
| ***Varroa destructor*-*defensin*** | -0.354 | 0.013 | 57 |
| **BQCV-*domeless*** | -0.334 | 0.011 | 57 |
| **BQCV-*dorsal*** | -0.277 | 0.039 | 57 |
| **DWV-*dorsal* (spring-summer season)** | -0.509 | 0.003 | 28 |

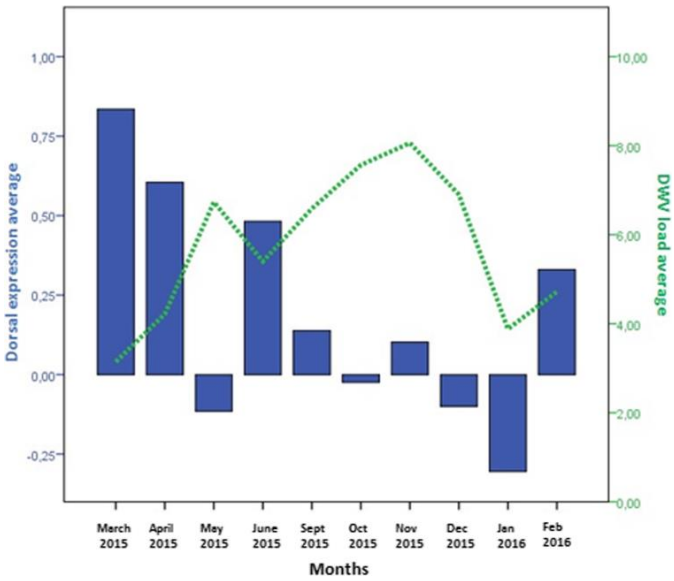


**Figure 4**. Comparison of mean *dorsal* expression (blue, left axis) and mean DWV load expressed as RNA equivalents/µl (green, right axis)

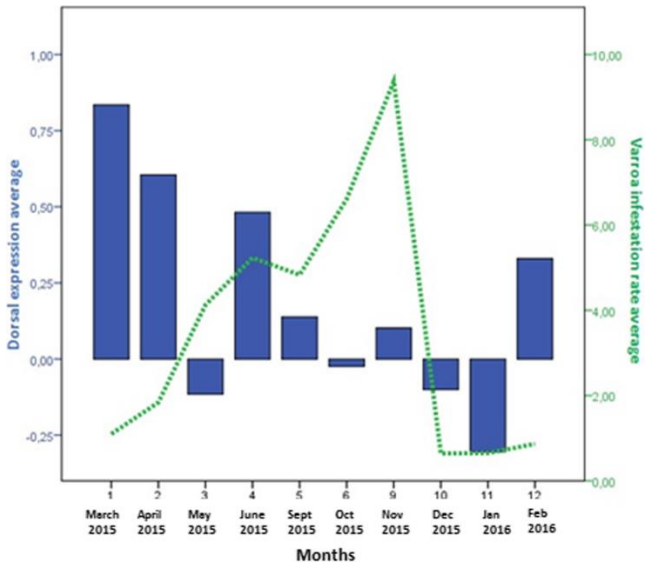


**Figure 5**. Comparison of mean *dorsal* expression (blue, left axis) and *Varroa destructor* infestation rate (green, right axis)

Analysis of the four colonies that collapsed during the study period (colonies 2, 3, 4, 5) revealed some trends. Correlations are shown in Table 1. All four colonies showed an increase in *relish* expression in the months before collapse, concomitant with increasing DWV and mite loads. In fact, *relish* expression peaked just before collapse of colonies 4 and 5, when DWV load also peaked. Although no significant relationships were observed between immune system gene expression and pathogen load, we did observe that relish expression generally tracked with DWV and mite loads, while *defensin* was expressed at lower levels in collapsed colonies than in non-collapsed ones. Results are plotted for every collapsing colony in Figure 6.

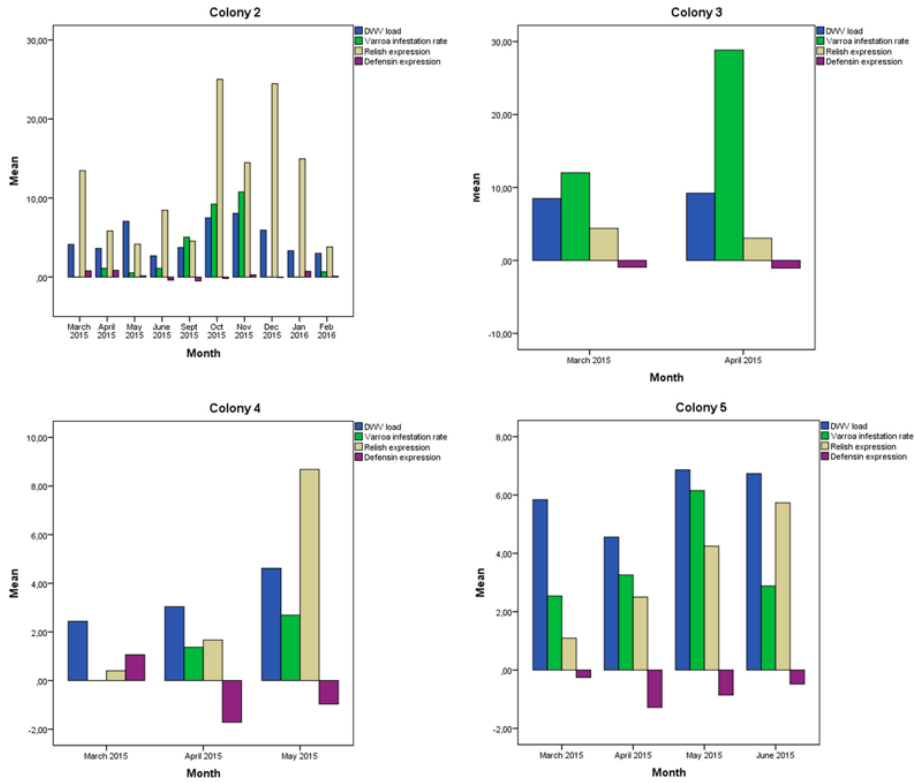


**Figure 6.** Comparison of DWV load, *Varroa destructor* infestation rate, and expression levels of *relish* and *defensin* in collapsing colonies. Y axis represents mean value for each variable. *Relish* and *defensin* expression was calculated as described in the legend to Fig. 2.

### 2.3.7 Immune system gene expression over time

Analysis of trends in immune system gene expression over the 12 months of the study period (Figure 2) showed that *relish* expression was significantly higher during the spring-summer than during autumn-winter (May-September 2015, Mann-Whitney *U* test, p=0.002), and the same was observed for *domeless* expression (Mann-Whitney *U* test, p=0.007). Similar seasonality was also observed for DWV load (Mann-Whitney *U* test, p=0.007) and *Varroa destructor* infestation rate (Mann-Whitney U test, p<0.001).

*Defensin* expression was higher during winter months than during spring-summer months, peaking in January 2016. In this way, *defensin* expression was higher when DWV load and *Varroa destructor* infestation were lower. No clear seasonality was observed in the expression of *dorsal*.

### 2.3.8 Comparison between collapsing and surviving colonies

We examined whether DWV load, BQCV load, *Varroa destructor* infestation rate, or expression of any of the four immune system genes differed significantly between the four colonies that collapsed and the remaining three colonies that did not. No significant relationships were found determined based on the Mann-Whitney *U* test. A tendency was observed in the case of *defensin* expression ($p=0.059$). Comparisons between collapsing and surviving colonies are plotted in figures 7 and 8.

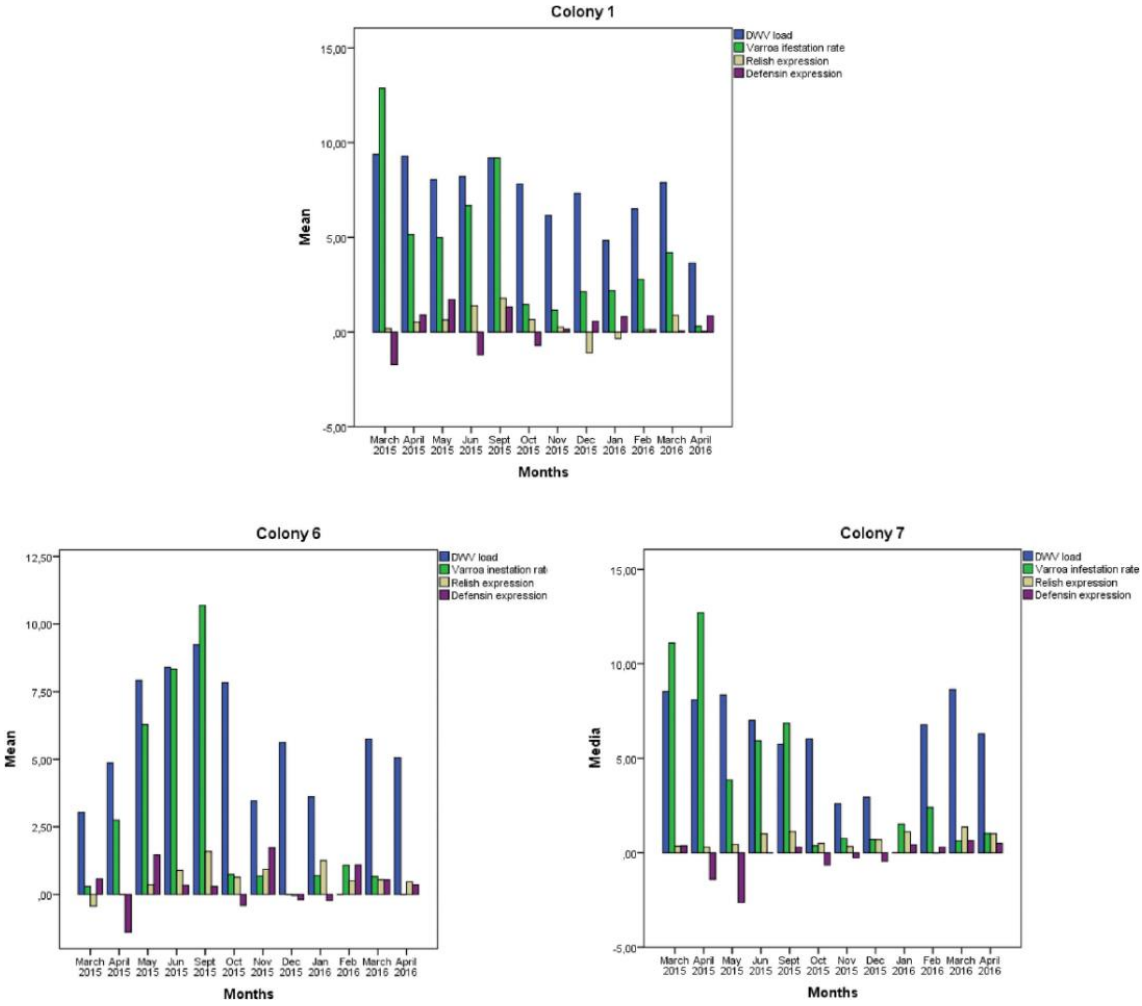


**Figure 7.** Comparison of DWV load, *Varroa destructor* infestation rate, and expression levels of *relish* and *defensin* in surviving colonies. Y axis represents mean value for each variable. *Relish* and *defensin* expression was calculated as described in the legend to Fig. 2

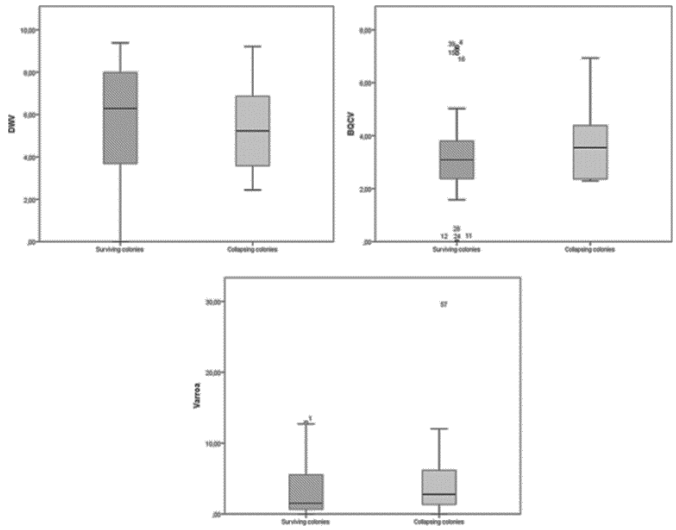


**Figure 8.** Differences in DWV load, BQCV load and *Varroa destructor* infestation rate between surviving and collapsing colonies. The analysis was performed using data from surviving and collapsing colonies during the whole period of study

# 2.4 Discussion

Here we provide evidence that monitoring expression of the *relish* and *defensin* genes may contribute to a better understanding of honey bees immune system and to assess pathogen load in the colony and, simultaneously, the capacity of the colony to resist those pathogens. To identify any potential immune marker, we screened for possible associations between levels of expression of four genes reflecting four major immune response pathways and load of DWV and *Varroa destructor*. The synergistic relationship between these two pathogens is one of the most challenging problems for beekeeping (Dainat et al., 2012b; Gisder et al., 2009; Shen, Yang, et al., 2005). Whether these preliminary results are validated in future studies, determination of *relish* and *defensin* may be useful for monitoring the health of colonies exposed to these pathogens. It may also be suitable for monitoring health in the face of other infectious diseases and immunosuppressive factors. Our study demonstrates, for the first time, the feasibility of monitoring bee colony health by

screening immune system gene expression instead of simply detecting pathogen presence. Most of the previous studies of honey bee immune response to pathogens were based on laboratory experiment. However, little is known about the immune response of honey bees naturally affected by DWV and *Varroa destructor*. In fact, the use of immune genes as markers for colony health to the field level has rarely been studied. Therefore, the present study set out with the aim of assessing the value of four immune genes as colony health markers.

How viral infection, stress, and season affect the honey bee immune system is not well understood, and details of the complex immune response to pathogens such as DWV and *Varroa destructor* need to be elucidated. We began to approach these questions by examining four genes involved in major immune responses to pathogens: *relish* is involved in the Imd pathway; *defensin*, in the production of antimicrobial peptides; *domeless*, in the Jak-STAT pathway; and *dorsal*, in the Toll pathway (Brutscher, Daughenbaugh, & Flenniken, 2015). We determined that increases in *relish* expression were closely linked to high DWV and mite loads. We also determined that decreases in *defensin* expression were correlated with high level of pathogens. In fact, collapsing colonies showed a decrease in *defensin* expression during the months prior to collapse. In this way, high *relish* expression may be an immune marker of DWV and mite load, and the combination of this up-regulation and *defensin* down-regulation may indicate that a colony is likely to experience difficulty in dealing with these pathogens and is therefore at risk of collapse. Therefore, the applicability of our findings to field conditions could be promising. However, our data must be interpreted with caution because of the small number of samples. Could we monitor individual colony health status through immune gene analysis? This question is not easy to answer, since every colony acts as a superorganism. Moreover, its immune system can vary in every particular case (for example, different weather conditions or pesticide exposure). For this reason, more research on this field would be very valuable to fully understand the role of immune

system in colony's health. Our results try to shed light to this field of research, these preliminary results showed the potential of this new approach for future research.

### 2.4.1 *Varroa destructor* and DWV are related to colony health status

Colonies showed high prevalence of DWV and BQCV, which in many cases were present as covert infections because they did not cause any apparent damage. This observation underscores that merely detecting these pathogens is inadequate for accurately assessing risk of colony collapse. It also suggests that DWV and the honey bee can enter into a sort of balance compatible with colony health. However, the combination of high DWV load and high *Varroa destructor* infestation rate can seriously undermine colony health, as observed in colonies 3-5. This is consistent with the idea that the mite acts as an immunosuppressive factor to trigger DWV replication and induce overt infection and, ultimately, colony collapse (Nazzi et al., 2012). This may explain the strong correlation observed between the virus and the mite in our study, which showed a seasonal pattern, with higher risk between May and November. This seasonality has been observed in previous studies (E. Genersch et al., 2010). Our analysis of immune responses confirms the proposal that quantitative analyses can help elucidate the dynamics of pathogen-host relationships (Gauthier et al., 2007; Genersch et al., 2010).

IAPV and SBV were not detected in any colony during the entire study. Although one study has associated IAPV with colony collapse disorder (Cox-Foster et al., 2007), more recent work in the same and other geographic areas as the present study suggests that this virus by itself is not a determinant in colony collapse (Vicente-Rubiano et al., 2013).

### 2.4.2 *Relish* as a predictive marker of DWV-*Varroa destructor* infection

*Relish* expression increased with DWV-mite load during the summer-autumn season. This likely reflects that DWV infection activates the Imd pathway, leading to NF-KB activation to deal with viral diseases. This NF-KB induction may have helped the colony survive despite high DWV infection. At the same time, our results suggest that the Imd pathway may be compromised: *relish*, like *dorsal* (Toll pathway), regulates the expression of several antimicrobial peptides, yet DWV and mite loads correlated negatively with *defensin*, which can serve as an index of antimicrobial peptide production.

One explanation for this finding is that the imbalance between DWV infection (mostly boosted by viral replication and transmission *Varroa destructor*) and host immune response inhibits the activity of effector molecules of the NF-KB family. An alternative explanation is that the Toll pathway, rather than the Imd pathway, may control *defensin* expression as was reported by Schlüns and Crozier (Schluns & Crozier, 2007). However, we did not find a positive correlation between *dorsal* and *defensin* expression; instead, we observed a positive correlation between *defensin* and *relish* expression.

Whatever the implications of our results for interactions between pathogens and the honey bee immune response, our data suggest that *relish* expression may serve as an immune indicator of colony health status. An increase in *relish* expression may reflect high DWV load and high *Varroa destructor* infestation rate, which can predispose the colony to collapse. However, further work is required to establish this.

### 2.4.3 *Defensin* as a predictive marker of colony health status

Like *relish* expression, *defensin* expression was closely, although negatively, associated with DWV and mite loads. This fact may reflect an immunosuppression in the production of this AMP. Collapsing colonies showed an even greater extent of decrease of *defensin* expression in the presence of high pathogen load than surviving colonies did. These findings may reflect the ability of *Varroa destructor* to down-regulate *defensin* expression (Khongphinitbunjong et al., 2015), potentially by promoting DWV replication. However, in a recent study performed by Zanni et al. (2017) (Zanni, Galbraith, Annoscia, Grozinger, & Nazzi, 2017), honey bees from high varroa-infested colonies showed up-regulation of two genes, GB51223 and GB51306. Both of them are involved in the production of two AMPs (*hymenoptaecin,* and *apidaecin*), whose up-regulation had been observed in presence of *Varroa destructor* previously (Kuster et al., 2014). Viral infection has also been reported to modulate levels of AMPs, but the underlying mechanism is still poorly understood (Danihlík, Aronstein, & Petřivalský, 2015).

We determined that *defensin* expression continuously decreased in colonies that collapsed, while it increased in colonies that also showed high pathogen loads and survived. This suggests that the combination of *defensin* expression and decreases in *relish* expression may mean that a colony is unlikely to survive an existing infection with DWV and the mite. This promising finding should be confirmed in further studies.

### 2.4.4 *Dorsal* expression negatively correlated with DWV infection

We observed a negative correlation between DWV load and *dorsal* expression during the spring-summer. This may reflect the ability of DWV to suppress the Toll immune pathway (Khongphinitbunjong et al., 2015; Van Rij et al., 2006), and this

suppression should be stronger in the summer, when *Varroa destructor* reproduction and therefore DWV replication increase. The combination of immunosuppression by the DWV-*Varroa destructor* complex and another stressor (e.g. nutritional or climatic) may render colonies more susceptible to collapse. Nevertheless, our results suggest that the Toll pathway can maintain acceptable colony health even in the presence of high DWV load, even on the order of $10^9$ RNA equivalents/µl. Indeed, we observed that higher *dorsal* expression was associated with lower DWV load. Further work should explore whether *dorsal* expression can reflect the ability of honey bee colonies to resist pathogens.

Like DWV load, BQCV load correlated negatively with *dorsal* expression, supporting the idea that high viral load suppresses the Toll pathway. BQCV usually persists in the colony at a low level, without causing apparent symptoms; its replication can be activated under certain circumstances, and colonies can survive even in the presence of high BQCV load. BQCV, as an opportunistic pathogen, is likely to contribute to colony losses only in combination with other factors (Khongphinitbunjong et al., 2015). BQCV load in our study peaked in spring, although its replication usually peaks in summer (Williams et al., 2009).

BQCV load also negatively correlated with *domeless* expression, probably reflecting the association between colony stress and inhibition of the Jak-STAT pathway (Tentcheva et al., 2004).

### 2.4.5 Assessing colony health with immune markers rather than pathogen load

The use of *relish* and *defensin* as immune markers may be useful for monitoring colony health, and it merits further study in larger field trials. If it can be validated, it would present several advantages over the use of DWV or mite load for assessing colony health. First, the dual marker can simultaneously provide information about (1) DWV-*Varroa destructor* infection and (2) whether the colony is

likely to survive despite the infection. Second, *relish* and *defensin* expression can be measured in a single quantitative PCR assay, which is simpler and more straightforward than determining DWV load and *Varroa destructor* infestation. Third, the immune markers may be useful for various infectious diseases and stress conditions, not only for DWV and *Varroa destructor.*

# 2.5 Conclusions

We have provided evidence that expression analysis of the immune system genes *relish* and *defensin* may be useful for monitoring colony health status, allowing us to develop new strategies to evaluate colony health in the field.

We determined that *relish* expression may serve as an indicator of DWV-*Varroa destructor* infection, and may in fact contribute to high pathogen load. We also determined that *defensin* expression may serve as an indicator of how well a colony is likely to resist an existing infection of DVW-*Varroa destructor*. The use of immune genes as biomarkers may allow us to establish new strategies to control DWV infection and *Varroa destructor* infestation. Improve monitoring at field level may be useful for identifying colonies in more urgent need of control measures, before significant damage has been occurred. Although we have analysed these genes in relation to the DWV-*Varroa destructor* complex, they may also be useful for preventing and controlling other infectious diseases. In addition, our study describes an approach for exploring differences in immune system genes as a function of DWV load and *Varroa destructor* infestation, which can help clarify the mechanisms of colony collapse.

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# Immune related genes as markers for monitoring health status of honey bee colonies

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## Abstract

**Background:** Honey bee population decline threatens the beekeeping sector, agriculture and global biodiversity. Early detection of colony mortality may facilitate rapid interventions to contain and prevent mortality spread. Among others, deformed wing virus (DWV) is capable of inducing colony losses, especially when combined with *Varroa destructor* mite. Since the bee immune system plays a crucial role in ensuring that bees are able to face these pathogens, we explored whether expression of immune genes could serve as biomarkers of colony health.

**Results:** Herein, we describe a preliminary immunological marker composed of two immune genes (*relish* and *defensin*), which provide insight on honey bee antiviral defense mechanism. Of the tested genes, *relish* expression correlated with the presence of DWV-Varroa complex, while decreased *defensin* expression correlated with poor resistance to this complex.

**Conclusions:** The monitoring of these genes may help us to better understand the complex physiology of honey bees's immune system and to develop new approaches for managing the health impacts of DWV infection and varroa infestation in the field.

**Keywords:** Honey bees, Deformed wing virus, Varroa, Immune system, Markers

## Background

The western honey bee *Apis mellifera* plays a critical role in pollination of important crops, but high annual losses in the US [1, 2] and over-wintering colony losses in Europe have had significant negative consequences on the environment and economy [3]. Both of these depopulation processes are poorly understood and are thought to be caused by multiple factors, such as high levels of pathogens, parasites, environmental pollutants, nutritional stress, inadequate beekeeping management and climate change [4, 5]. Generally speaking, pesticides and pathogens have been reported to be important factors contributing colony losses. The available evidence seems to suggest that collapsing and weak colonies have a greater prevalence of pathogens compared to healthy colonies [6]. On the other hand, laboratory studies have demonstrated that exposure to sub lethal doses of pesticides can negatively affect honey bee behaviour [7, 8], foraging [9] and longevity [10]. However, only neonicotinoid exposure has been reported to act synergistically with pathogens, by reducing immune defences and promoting the replication of the DWV in honey bees [11].

Several pathogens and parasites have been associated with honey bee colony losses, especially the *Varroa destructor* mite and deformed wing virus (DWV), which have been described as predictive markers of winter losses [12, 13]. These two agents are interrelated: *Varroa destructor* harms colonies directly by feeding on honey bee haemolymph, and it harms colonies indirectly by facilitating the transmission of DWV and other viruses. In addition to viral transmission, immunosuppression of the developing honeybee by *Varroa destructor* has been suggested to explain the synergetic relationship between DWV and the mite. However, a recent study carried out

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by Kuster et al. (2014) [14] revealed that mite feeding activity itself and not immunosuppression may be the cause of this synergy. Several studies have associated this mite-DWV interaction to increased risk of winter losses [14] As for DWV, different genetic variants have been described [15, 16]. In fact, the mite may even drive selection for more pathogenic variants of DWV, increasing the likelihood of colony collapse [17–19].

These results suggest that assaying levels of *Varroa destructor* or DWV in a colony may predict colony death. However, colonies have been shown to survive even in the presence of high DWV load [20]. Therefore, being able to distinguish between a normal situation and a pathogenic one is crucial for establishing a proper colony monitoring. As reported by Nazzi et al. (2018) [13], the molecular analyses have revealed that the immune system of honey bees may be determinant in the modulation of this synergistic association. An immune-suppressive syndrome, characterized by a negative transcriptional regulation of several genes, may drive the conversion from "covert" to "overt" infection. This immune suppression can easily trigger colony mortality [21], since the immune system of individual bees plays a key role in colony health status [22, 23] together with colony-level anti-pathogen measures such as social hygiene and other colony-level behaviours [20].

Knowledge of honey bee immune mechanisms is mostly resulting via comparison to the better-characterized immune responses in fruit-flies and mosquitoes. General aspects of immunity, including detection of pathogen associated molecular patterns (PAMPs) and production of effector molecules are conserved in mammals, plants, and insects, and both plants and insects employ RNA interference (RNAi) as a major mechanism of antiviral defence [24, 25]. The individual innate response comprises a humoral and cellular immune response [26, 27]. Cellular response consists in phagocytosis, encapsulation and melanization mechanisms [28]. Both nodulation and encapsulation are frequently accompanied with melanization, which are catalysed by pro-Phenoloxidase (PO) [29]. The humoral response involves secretion of antimicrobial peptides, melanisation, and the enzymatic degradation of pathogens [30]. The innate immune system in honey bees is composed of pattern recognition receptors (PRRs) that interact with pathogen-associated molecular patterns (PAMPs), stimulating different pathways as a function of each type of pathogen. Gram-positive bacteria and/or fungi are thought to stimulate both the Toll pathway, leading to up-regulation of *dorsal*, and the Immune Deficiency (Imd) pathway, leading to up-regulation of *relish* [31]. Viruses, for example, trigger mainly the RNA interference pathway [32, 33], although DWV infection in honey bees also down-regulates *dorsal*, suggesting inhibition of the Toll pathway [34]; in fact, RNAi mediated silencing of this gene was clearly associated with increased viral replication [13].

Thus, there is evidence that the immune system plays a crucial role in ensuring colony survival and that honey bees have innate immune mechanisms to fight against infections that have been related to colony mortality [23]. However, although advances in elucidating these immune mechanisms have been reached in last years, it is not fully understood how particular infections trigger complex responses in colonies and how these responses evolve throughout the seasons. Hence, thorough studies of the biological significance of most genes in vivo are required.

The present study explored whether expression levels of four immune system genes could serve as biomarkers of elevated risk of colony mortality. This preliminary immune marker was defined in relation to the worst pathogen scenario that honey bees have to face: the joint action of *Varroa destructor* and DWV, which may be extrapolated to other infectious diseases. In seven honey bee colonies in one apiary in Spain, we examined possible correlation of *Varroa destructor* and DWV load with expression of four *A. mellifera* genes involved in honey bee immunity and colony health status for ten months. Both pathogens have been described as predictive markers of honey bee colony collapse [12] and their monitoring may help beekeepers to establish preventive measures. However, they do not provide enough information about colony health status, since the colony is able to deal with infectious pathogens on many times if its immune system works properly. Thus, this study describes a preliminary marker based on immune system response, which provides not only information about pathogens affecting the colony, but also of how the colony is facing them. This marker is based on evaluating relish and *defensin* expression through qPCR analysis. *Relish* expression reflects levels of DWV infection and varroa infestation, while *defensin* expression reflects how well the colony can resist these pathogens. To monitor such double immune marker in the most critical moments (winter, extreme temperatures, high *Varroa destructor* infestation, risk management) would help beekeepers to set up preventive measures and to standardize honey bee colony monitoring. However, further studies should be conducted to test the application of this double immune marker under different environmental conditions.

## Results

Four colonies collapsed during the study: colony 2 (May 2016), colony 3 (May 2015), colony 4 (June 2015) and colony 5 (July 2015).

### Viral load

IAPV and SBV were not detected in any sample. DWV was more prevalent than BQCV throughout the study

period. Nearly all samples (97.5%) were positive for DWV, with load ranging from $2.75 \times 10^2$ to $2.39 \times 10^9$ RNA equivalents/μl. Colony 3 had the highest mean DWV load ($2.39 \times 10^9$ RNA equivalents/μl), and it rapidly collapsed before the summer. Colony 1 had the second-highest mean DWV load. Both colonies also showed the highest rates of *Varroa destructor* infestation, at 20.43 and 4.42%, respectively. Mean DWV load was $2.04 \times 10^5$ GEC/μl during the winter and $2.69 \times 10^6$ RNA equivalents/μl during the warmer months.

BQCV showed a mean prevalence of 89.43% and mean load of $1.66 \times 10^3$ RNA equivalents/μl. Colony 4 showed the highest mean BQCV load ($4.37 \times 10^3$ RNA equivalents/μl), followed by colony 6 ($3.72 \times 10^3$ RNA equivalents/μl). Mean BQCV load was $2.99 \times 10^2$ during the winter and $2.88 \times 10^3$ during the warmer months.

### Varroa destructor infestation

In colonies that survived until the end of the study, the mite was present for at least 9 of the 12 samplings, and infestation rates varied from 0.3 to 28.85% (Fig. 1). *Varroa destructor* infestation rates were higher in warmer months (May, June, and September) and lower in autumn and winter months, following acaricide treatment. The rate dropped significantly between September and October following oxalic acid treatment ($p = 0.021$, Mann Whitney test). Colony 3 showed *Varroa destructor* infestation rates > 10% in March and April 2015, and it collapsed in May 2015. The combination of high mite infestation and high DWV load may be the primary causes of the collapse.

*Varroa destructor* infestation rate correlated with DWV load across all seven colonies over the entire study period ($r_s = 0.648$, $p < 0.001$), as well as specifically in colonies 1 ($r_s = 0.829$), 4 ($r_s = 1$) and 7 ($r_s = 0.648$, all $p < 0.05$).

### Nosema ceranae infection

*Nosema ceranae* was not detected in any sample.

### Correlations among the immune pathways studied

Comparison of levels of expression of the four immune system genes from three immune response pathways (Fig. 2) revealed three positive correlations among the pathways (Table 1). One correlation occurred between relish and *defensin* ($r_s = 0.405$, $p = 0.002$), reflecting the production of antimicrobial peptides via the Imd pathway. Another correlation occurred between *relish* and

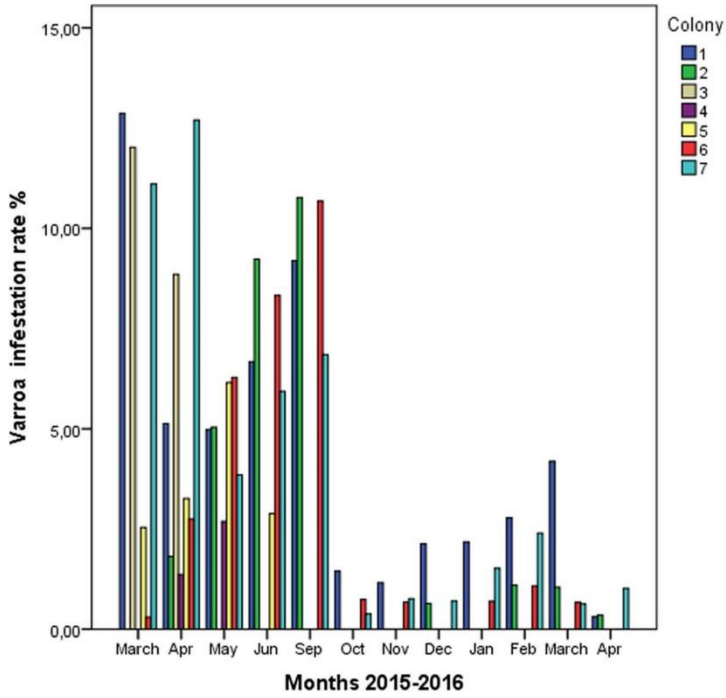


**Fig. 1** Rates of *Varroa destructor* infestation (per 100 adult bees) in each colony based on monthly sampling

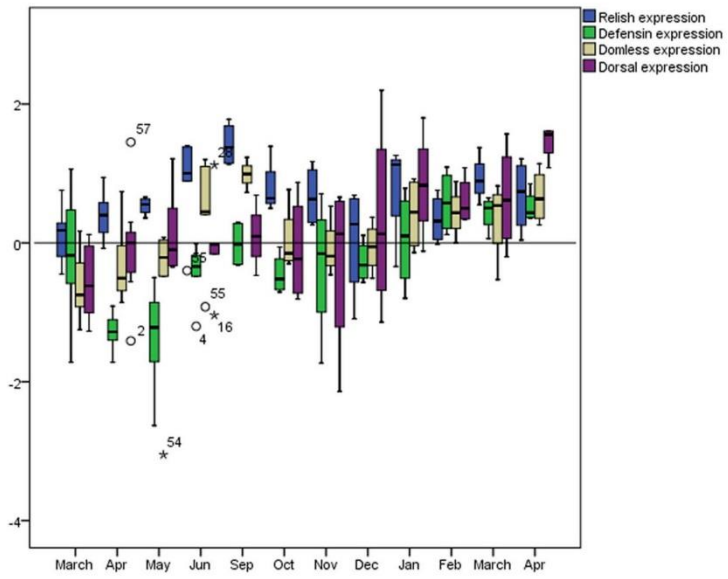


**Fig. 2** Relative expression of immune system genes across all seven colonies in the apiary during the study period. Expression levels are expressed relative to adjusted for the levels observed in uninfected reference samples. The solid horizontal line indicates relative expression equal to that in the uninfected reference

*domeless* ($r_s = 0.707$, $p < 0.001$), reflecting the fact that the Jak-STAT pathway is activated by viruses and Gram-negative bacteria, although we did not test bacterial load. A third correlation was observed between *domeless* and *defensin* ($r_s = 0.422$, $p = 0.001$).

### Apiary-level analysis of immune response to DWV infection and *Varroa destructor* infestation

Expression of *defensin* correlated negatively with DWV load ($r_s = -0.385$, $p = 0.008$) and mite load ($r_s = -0.354$, $p = 0.13$). Expression of *domeless* correlated negatively with BQCV load ($r_s = -0.334$, $p = 0.011$). Expression of *dorsal* correlated negatively with BQCV load across the study period ($r_s = -0.277$, $p = 0.039$) and with DWV load during the spring-summer season ($r_s = -0.509$, $p = 0.003$).

**Table 1** Correlations in relative expression levels between pairs of immune system genes, based on the data shown in Fig. 2

| Interaction | Correlation coefficient | p | n |
|---|---|---|---|
| *Relish-defensin* | 0.395 | 0.002 | 57 |
| *Relish-domeless* | 0.702 | < 0.001 | 57 |
| *Defensin-domeless* | 0.630 | < 0.001 | 57 |
| *Defensin-dorsal* | 0.539 | < 0.001 | 57 |
| *Domeless-dorsal* | 0.470 | < 0.001 | 57 |

Conversely, expression of *relish* correlated positively with DWV load during the spring-summer season ($r_s = 0.403$, $p = 0.042$).

In addition to these analyses in which DWV load was treated as a continuous variable, we analysed the load in categorical terms of low or high. Expression of *dorsal* was significantly lower in colonies with high load than in those with low load (Mann-Whitney *U* test, $p = 0.013$). Conversely, expression of *relish* was significantly higher in colonies with high DWV load than in those with low load (Mann-Whitney *U* test, $p = 0.049$).

### Colony-level analysis of immune response to DWV infection and *Varroa destructor* infestation

Significant relationships between immune system gene expression and pathogen load were detected within individual colonies (Table 2). High DWV and mite loads were usually associated with an increase in *relish* expression (Fig. 3) but with a decrease in *dorsal* and *defensin* expression (Figs. 4 and 5). In fact, in colonies 1, 6 and 7, which survived the entire study period, an increase of *dorsal* expression in the winter was accompanied by a decrease of DWV load. However, colonies 2 and 4 showed increased of *dorsal* expression in the month prior to collapse. In colony 2, *relish* expression

**Table 2** Correlations between pathogen load and immune system gene expression

| Interaction | Correlation coefficient | p | n |
|---|---|---|---|
| DWV-*defensin* | −0.385 | 0.008 | 57 |
| *Varroa destructor*-*defensin* | −0.354 | 0.013 | 57 |
| BQCV-*domeless* | −0.334 | 0.011 | 57 |
| BQCV-*dorsal* | −0.277 | 0.039 | 57 |
| DWV-*dorsal* (spring-summer season) | −0.509 | 0.003 | 28 |

All correlation analyses were performed using data from the whole period of study, with exception of the DWV-*dorsal* correlation, which was analysed using data from the spring-summer season

correlated positively with *domeless* expression ($r = 0.909$, $p < 0.001$) and *dorsal* expression ($r = 0.783$, $p = 0.003$).

Analysis of the four colonies that collapsed during the study period (colonies 2, 3, 4, 5) revealed some trends. Correlations are shown in Table 3. All four colonies showed an increase in *relish* expression in the months before collapse, concomitant with increasing DWV and mite loads. In fact, *relish* expression peaked just before collapse of colonies 4 and 5, when DWV load also peaked. Although no significant relationships were observed between immune system gene expression and pathogen load, we did observe that *relish* expression generally tracked with DWV and mite loads, while *defensin* was expressed at lower levels in collapsed colonies than in non-collapsed ones. Results are plotted for every collapsing colony in Fig. 6 and for every surviving colony in Fig. 7.

### Immune system gene expression over time

Analysis of trends in immune system gene expression over the 12 months of the study period (Fig. 2) showed that *relish* expression was significantly higher during the spring-summer than during autumn-winter (May–September 2015, Mann-Whitney *U* test, $p = 0.002$), and the same was observed for *domeless* expression (Mann-Whitney *U* test, $p = 0.007$). Similar seasonality was also observed for DWV load (Mann-Whitney *U* test, $p = 0.007$) and *Varroa destructor* infestation rate (Mann-Whitney *U* test, $p < 0.001$).

*Defensin* expression was higher during winter months than during spring-summer months, peaking in January 2016. In this way, *defensin* expression was higher when DWV load and *Varroa destructor* infestation were lower. No clear seasonality was observed in the expression of *dorsal*.

### Comparison between collapsing and surviving colonies

We examined whether DWV load, BQCV load, *Varroa destructor* infestation rate, or expression of any of the four immune system genes differed significantly between

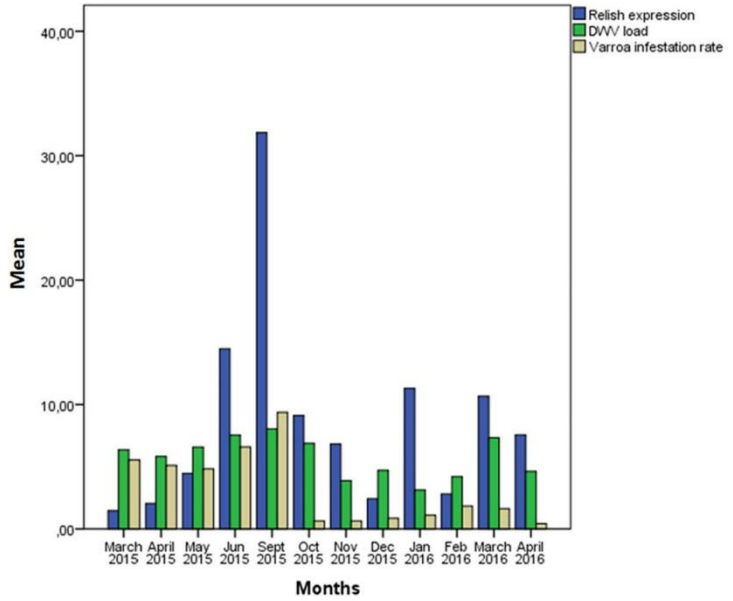


**Fig. 3** Comparison of relative *relish* expression, DWV load and *Varroa destructor* infestation rate. The y-axis shows mean values for all seven colonies in the apiary. Relish expression was calculated as described in the legend to Fig. 2

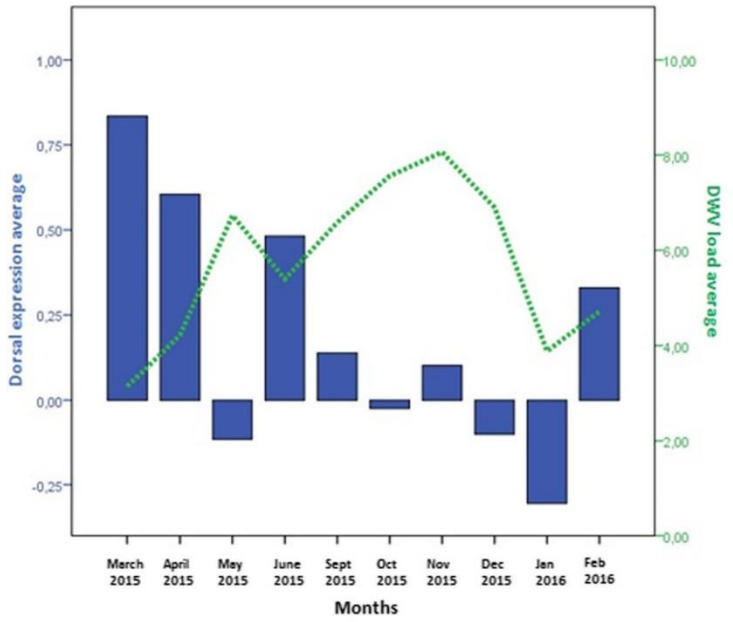


**Fig. 4** Comparison of mean *dorsal* expression (blue, left axis) and mean DWV load expressed as RNA equivalents/μl (green, right axis)

the four colonies that collapsed and the remaining three colonies that did not. No significant relationships were found determined based on the Mann-Whitney *U* test. A tendency was observed in the case of *defensin* expression ($p = 0.059$). Comparisons between collapsing and surviving colonies are plotted in Figs. 8 and 9.

## Discussion

Here we provide evidence that monitoring expression of the *relish* and *defensin* genes may contribute to a better understanding of honey bees immune system and to assess pathogen load in the colony and, simultaneously, the capacity of the colony to resist those pathogens. To identify any potential immune marker, we screened for possible associations between levels of expression of four genes reflecting four major immune response pathways and load of DWV and *Varroa destructor*. The synergistic relationship between these two pathogens is one of the most challenging problems for beekeeping [12, 35, 36]. Whether these preliminary results are validated in future studies, determination of *relish* and *defensin* may be useful for monitoring the health of colonies exposed to these pathogens. It may also be suitable for monitoring health in the face of other infectious diseases and immunosuppressive factors. Our study demonstrates, for the first time, the feasibility of monitoring bee colony health by screening immune system gene expression instead of simply detecting pathogen presence. Most of the previous studies of honey bee immune response to pathogens were based on laboratory experiment. However, little is known about the immune response of honey bees naturally affected by DWV and *Varroa destructor*. In fact, the use of immune genes as markers for colony health to the field level has rarely been studied. Therefore, the present study set out with the aim of assessing the value of four immune genes as colony health markers.

How viral infection, stress, and season affect the honey bee immune system is not well understood, and details of the complex immune response to pathogens such as DWV and *Varroa destructor* need to be elucidated. We began to approach these questions by examining four genes involved in major immune responses to pathogens: *relish* is involved in the Imd pathway; *defensin*, in the production of antimicrobial peptides; *domeless*, in the Jak-STAT pathway; and *dorsal*, in the Toll pathway [37]. We determined that increases in *relish* expression were closely linked to high DWV and mite loads. We also determined that decreases in *defensin* expression were correlated with high level of pathogens. In fact, collapsing colonies showed a decrease in *defensin* expression during the months prior to collapse. In this way,

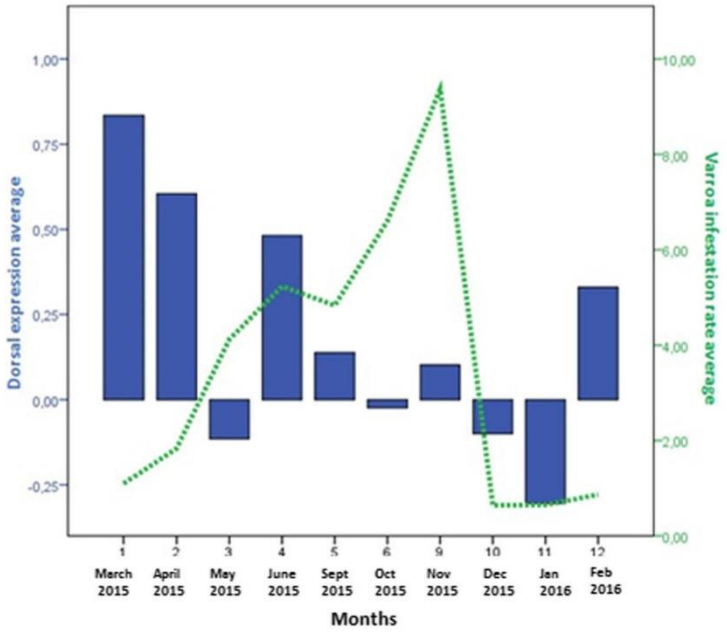


**Fig. 5** Comparison of mean *dorsal* expression (blue, left axis) and *Varroa destructor* infestation rate (green, right axis)

high *relish* expression may be an immune marker of DWV and mite load, and the combination of this up-regulation and *defensin* down-regulation may indicate that a colony is likely to experience difficulty in dealing with these pathogens and is therefore at risk of collapse. Therefore, the applicability of our findings to field conditions could be promising. However, our data must be interpreted with caution because of the small number of samples. Could we monitor individual colony health status through immune gene analysis? This question is not easy to answer, since every colony acts as a superorganism. Moreover, its immune system can vary in every particular case (for example, different weather conditions or pesticide exposure). For this reason, more research on this field would be very valuable to fully understand the role of immune system in colony's health. Our results try to shed light to this field of research, these preliminary results showed the potential of this new approach for future research.

### *Varroa destructor* and DWV are related to colony health status

Colonies showed high prevalence of DWV and BQCV, which in many cases were present as covert infections because they did not cause any apparent damage. This observation underscores that merely detecting these pathogens is inadequate for accurately assessing risk of colony collapse. It also suggests that DWV and the honey bee can enter into a sort of balance compatible with colony health. However, the combination of high DWV load and high *Varroa destructor* infestation rate can seriously undermine colony health, as observed in

**Table 3** Correlations of *relish* or *defensin* expression with DWV load or *Varroa destructor* infestation rate

| Collapsing colony | Correlations | | | |
|---|---|---|---|---|
| | *Relish*-DWV | *Relish-Varroa destructor* | *Defensin-DWV* | *Defensin-Varroa destructor* |
| Colony 2 | 0.397 ($p = 0.201$) | 0.298 ($p = 0.347$) | −0.143 ($p = 0.559$) | −0.229 ($p = 0.474$) |
| Colony 3 | 1 ($p = 0.164$) | 0.322 ($p = 0.678$) | −0.423 ($p = 0.338$) | **−0.999 ($p = 0.001$)** |
| Colony 4 | 0.992 ($p = 0.079$) | 0.925 ($p = 0.168$) | −0.505 ($p = 0.066$) | −0.712 ($p = 0.245$) |
| Colony 5 | 0.633 ($p = 0.164$) | 0.322 ($p = 0.678$) | −0.423 ($p = 0.338$) | **−0.999 ($p = 0.001$)** |

Correlation analysis was performed using data from two months prior to collapse. The significant correlations are marked in bold

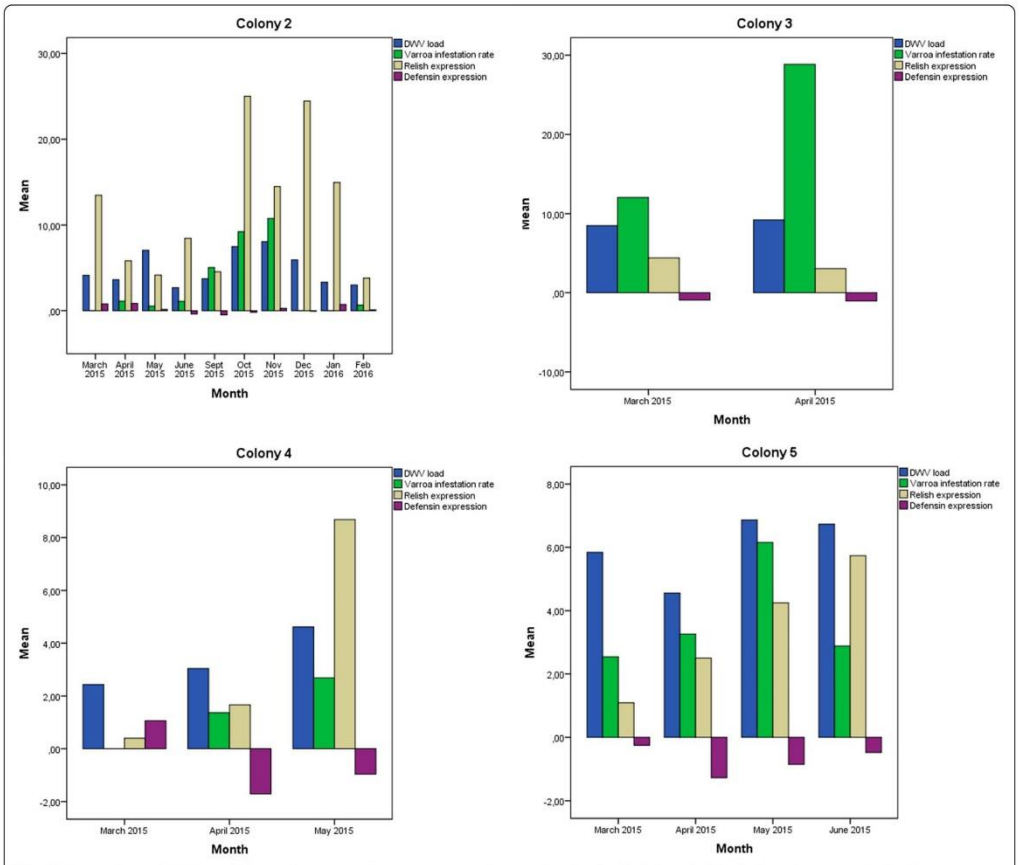


**Fig. 6** Comparison of DWV load, *Varroa destructor* infestation rate, and expression levels of *relish* and *defensin* in collapsing colonies. Y axis represents mean value for each variable. *Relish* and *defensin* expression was calculated as described in the legend to Fig. 2

colonies 3–5. This is consistent with the idea that the mite acts as an immunosuppressive factor to trigger DWV replication and induce overt infection and, ultimately, colony collapse [38]. This may explain the strong correlation observed between the virus and the mite in our study, which showed a seasonal pattern, with higher risk between May and November. This seasonality has been observed in previous studies [39]. Our analysis of immune responses confirms the proposal that quantitative analyses can help elucidate the dynamics of pathogen-host relationships [39, 40].

IAPV and SBV were not detected in any colony during the entire study. Although one study has associated IAPV with colony collapse disorder [41], more recent work in the same and other geographic areas as the present study suggests that this virus by itself is not a determinant in colony collapse [42].

### *Relish* as a predictive marker of DWV-*Varroa destructor* infection

*Relish* expression increased with DWV-mite load during the summer-autumn season. This likely reflects that DWV infection activates the Imd pathway, leading to NF-κB activation to deal with viral diseases. This NF-κB induction may have helped the colony survive despite high DWV infection. At the same time, our results suggest that the Imd pathway may be compromised: *relish*, like *dorsal* (Toll pathway), regulates the expression of several antimicrobial peptides, yet DWV and mite loads

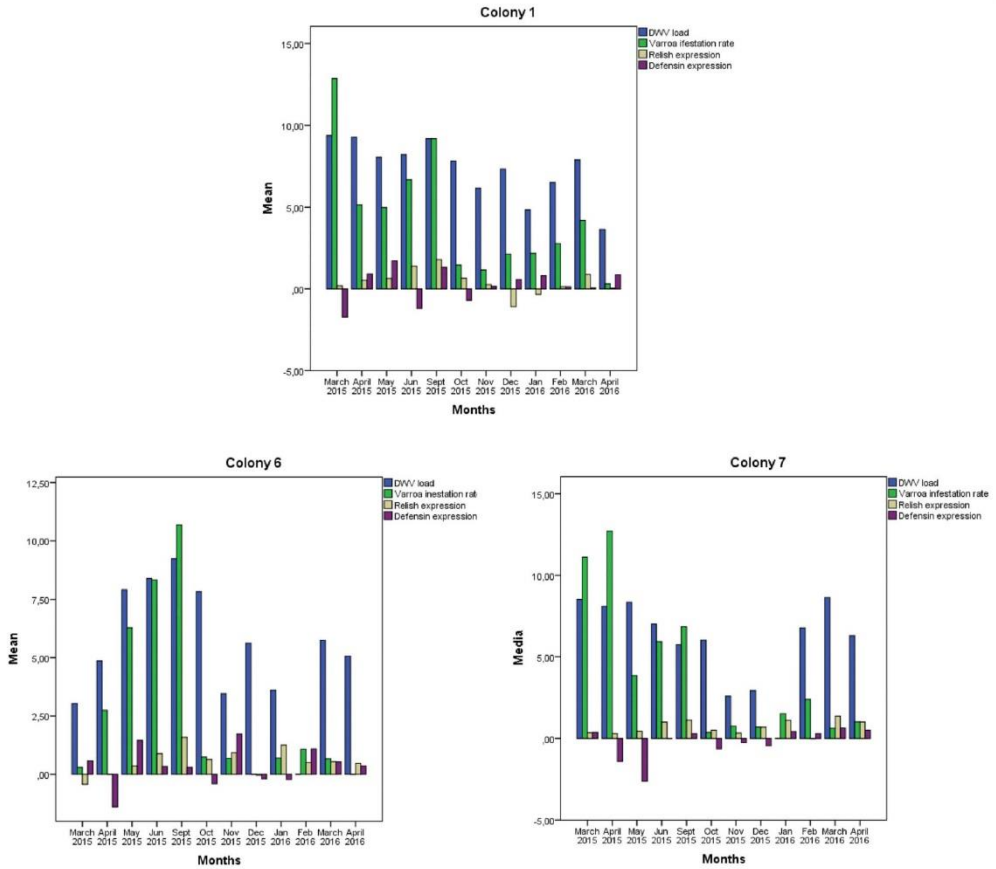


**Fig. 7** Comparison of DWV load, *Varroa destructor* infestation rate, and expression levels of *relish* and *defensin* in surviving colonies. Y axis represents mean value for each variable. *Relish* and *defensin* expression was calculated as described in the legend to Fig. 2

correlated negatively with *defensin*, which can serve as an index of antimicrobial peptide production.

One explanation for this finding is that the imbalance between DWV infection (mostly boosted by viral replication and transmission *Varroa destructor*) and host immune response inhibits the activity of effector molecules of the NF-κB family. An alternative explanation is that the Toll pathway, rather than the Imd pathway, may control *defensin* expression as was reported by Schlüns and Crozier [43]. However, we did not find a positive correlation between *dorsal* and *defensin* expression; instead, we observed a positive correlation between *defensin* and *relish* expression.

Whatever the implications of our results for interactions between pathogens and the honey bee immune response, our data suggest that *relish* expression may serve as an immune indicator of colony health status. An increase in *relish* expression may reflect high DWV load and high *Varroa destructor* infestation rate, which can predispose the colony to collapse. However, further work is required to establish this.

### *Defensin* as a predictive marker of colony health status

Like *relish* expression, *defensin* expression was closely, although negatively, associated with DWV and mite loads. This fact may reflect an immunosuppression in the production of this AMP. Collapsing colonies showed an even greater extent of decrease of *defensin* expression in the presence of high pathogen load than surviving colonies did. These findings may reflect the ability of *Varroa destructor* to down-regulate *defensin* expression

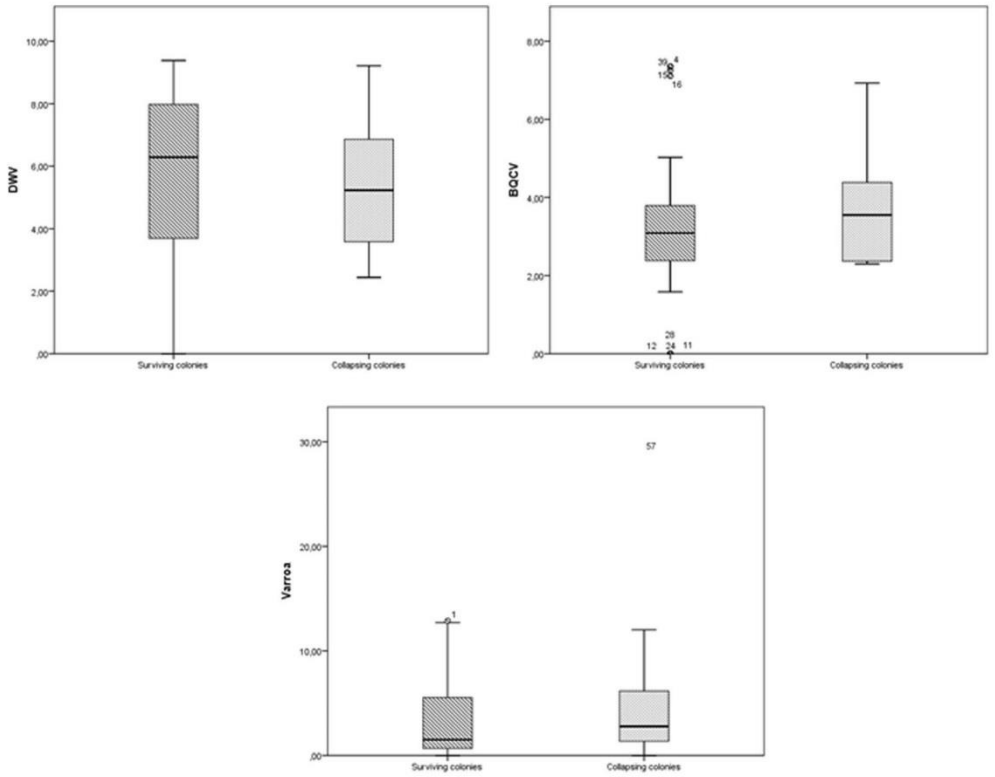


**Fig. 8** Differences in DWV load, BQCV load and *Varroa destructor* infestation rate between surviving and collapsing colonies. The analysis was performed using data from surviving and collapsing colonies during the whole period of study

[44], potentially by promoting DWV replication. However, in a recent study performed by Zanni et al. (2017) [45], honey bees from high varroa-infested colonies showed up-regulation of two genes, GB51223 and GB51306. Both of them are involved in the production of two AMPs (*hymenoptaecin*, and *apidaecin*), whose up-regulation had been observed in presence of *Varroa destructor* previously [14]. Viral infection has also been reported to modulate levels of AMPs, but the underlying mechanism is still poorly understood [46].

We determined that *defensin* expression continuously decreased in colonies that collapsed, while it increased in colonies that also showed high pathogen loads and survived. This suggests that the combination of *defensin* expression and decreases in *relish* expression may mean that a colony is unlikely to survive an existing infection with DWV and the mite. This promising finding should be confirmed in further studies.

### *Dorsal* expression negatively correlated with DWV infection

We observed a negative correlation between DWV load and *dorsal* expression during the spring-summer. This may reflect the ability of DWV to suppress the Toll immune pathway [34, 44], and this suppression should be stronger in the summer, when *Varroa destructor* reproduction and therefore DWV replication increase. The combination of immunosuppression by the DWV-*Varroa destructor* complex and another stressor (e.g. nutritional or climatic) may render colonies more susceptible to collapse. Nevertheless, our results suggest that the Toll pathway can maintain acceptable colony health even in the presence of high DWV load, even on the order of $10^9$ RNA equivalents/μl. Indeed, we observed that higher *dorsal* expression was associated with lower DWV load. Further work should explore whether *dorsal* expression can reflect the ability of honey bee colonies to resist pathogens.

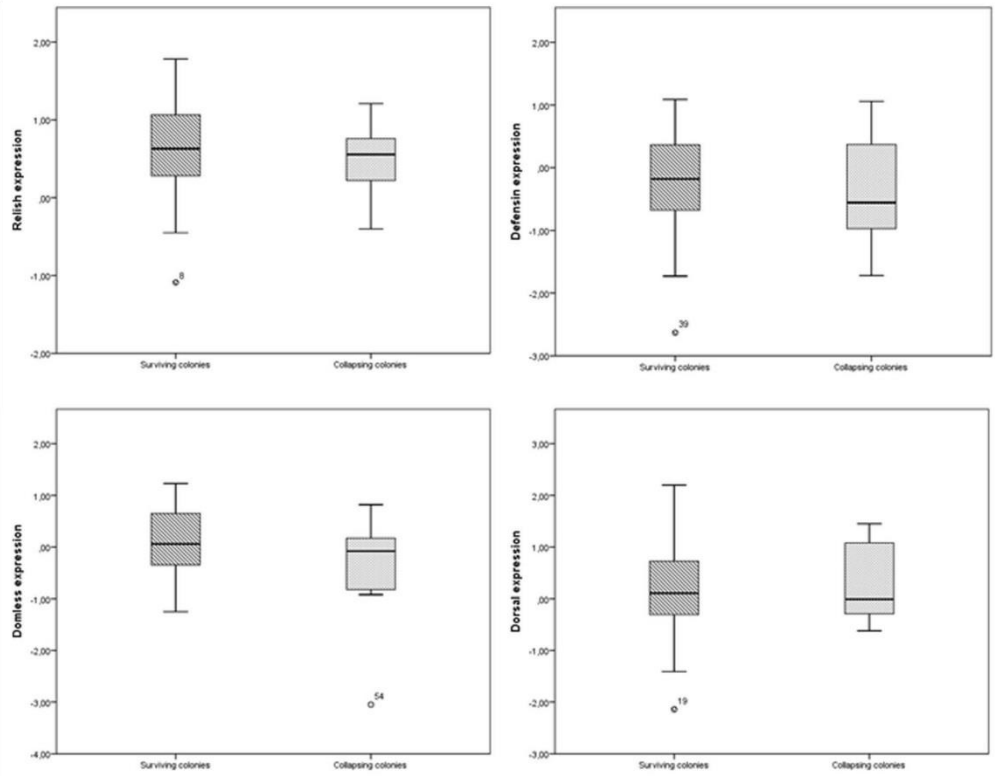


**Fig. 9** Differences in immune system gene expression between surviving and collapsing colonies. The analysis was performed using data from surviving and collapsing colonies during the whole period of study

Like DWV load, BQCV load correlated negatively with *dorsal* expression, supporting the idea that high viral load suppresses the Toll pathway. BQCV usually persists in the colony at a low level, without causing apparent symptoms; its replication can be activated under certain circumstances, and colonies can survive even in the presence of high BQCV load. BQCV, as an opportunistic pathogen, is likely to contribute to colony losses only in combination with other factors [44]. BQCV load in our study peaked in spring, although its replication usually peaks in summer [47].

BQCV load also negatively correlated with *domeless* expression, probably reflecting the association between colony stress and inhibition of the Jak-STAT pathway [48].

### Assessing colony health with immune markers rather than pathogen load

The use of *relish* and *defensin* as immune markers may be useful for monitoring colony health, and it merits further study in larger field trials. If it can be validated, it would present several advantages over the use of DWV or mite load for assessing colony health. First, the dual marker can simultaneously provide information about (1) DWV-*Varroa destructor* infection and (2) whether the colony is likely to survive despite the infection. Second, *relish* and *defensin* expression can be measured in a single quantitative PCR assay, which is simpler and more straightforward than determining DWV load and *Varroa destructor* infestation. Third, the immune markers may be useful for various infectious diseases and stress conditions, not only for DWV and *Varroa destructor*.

## Conclusions

We have provided evidence that expression analysis of the immune system genes *relish* and *defensin* may be useful for monitoring colony health status, allowing us to develop new strategies to evaluate colony health in the field.

We determined that *relish* expression may serve as an indicator of DWV-*Varroa destructor* infection, and may in fact contribute to high pathogen load. We also determined that *defensin* expression may serve as an indicator of how well a colony is likely to resist an existing infection of DVW-*Varroa destructor*. The use of immune genes as biomarkers may allow us to establish new strategies to control DWV infection and *Varroa destructor* infestation. Improve monitoring at field level may be useful for identifying colonies in more urgent need of control measures, before significant damage has been occurred. Although we have analysed these genes in relation to the DWV-*Varroa destructor* complex, they may also be useful for preventing and controlling other infectious diseases. In addition, our study describes an approach for exploring differences in immune system genes as a function of DWV load and *Varroa destructor* infestation, which can help clarify the mechanisms of colony collapse.

## Methods

### Experimental design

An experimental apiary of seven Langstroth hives of *Apis mellifera* was established in the Reference Centre for Beekeeping at the University of Cordoba (Cordoba, Spain). During the period from March 2015 to April 2016, all colonies were sampled monthly except for July and August, when sampling was impossible due to high temperatures in the apiary. At each sampling, a beekeeping technician inspected colonies; determined numbers of honey bees, brood, pollen and honey combs using the subjective method as described [49]; and noted the presence of symptoms, mortality and depopulation. Samples of approximately 50 adult bees were carefully taken by hand from the hive entrance or the honey combs of each colony and frozen at − 80 °C until analysis. Sampling process was systematically repeated among colonies in order to obtain the most homogenous sample under field conditions.

### Quantification of *Varroa destructor* load

*Varroa destructor* load was quantified monthly in all colonies throughout the study except for July and August 2015. Mite presence was assessed at each monthly sampling. Mite load was quantified using the soapy water method described in "Standard methods for varroa research" in the COLOSS BEEBOOK [50]. Briefly, 300 adult bees were collected from the colony from the sides of the unsealed brood combs, shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. The percentage of mites was calculated as follows:

$$\%\text{infestation} = (\text{no.mites}/\text{no.bees counted}) \times 100\%$$

After sampling and inspection in September 2015 and March 2016, colonies were treated with oxalic acid against the mite.

### RNA extraction

Ten intact bees were homogenized in 5 ml phosphate-buffered saline (PBS, pH 7.2) with mortar and pestle, and total RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel) according to the manufacturer's instructions. Total RNA was suspended in RNase/DNase-free water and stored at − 80 °C (RNA).

### Virus testing

RNA samples were assayed to determine load of four bee viruses: DWV, black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV) and sacbrood bee virus (SBV). One-step, real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad), SYBR Green detection and primers and cycling protocols previously published for DWV [51], BQCV [51], IAPV [52] and SBV [53].

Viral load of positive samples was determined by absolute quantification based on a standard curve constructed using serial 10-fold dilutions of known amounts of PGemT® TA plasmid (Promega) containing fragments of DWV and BQCV. Standard curves were fitted with lines showing correlation coefficients of 0.99 (data not shown). Viral loads were expressed in absolute terms in terms of RNA equivalents per microliter (RNA equivalents/μl), and in relative terms using a 4-point scale [53]: free of virus (RNA equivalents /μl = 0), low virus load ($0 < \text{RNA equivalents}/\mu l < 10^3$), medium virus load ($10^3 \leq \text{RNA equivalents}/\mu l < 10^7$) and high virus load ($\text{RNA equivalents}/\mu l \geq 10^7$). This procedure can detect virus that has infected at least 25% of a colony with 95% probability [54].

### Nosema cerana testing

To extract DNA for microsporidia, the protocol of BeeBook was adapted [55]. Two hundred μl of the homogenates used for virus testing were centrifuged at 16,100 g and supernatant was discarded. Pellets were frozen and crushed with sterile tips to disrupt nosema spores. This process was repeated three times before extraction of DNA with DNA Isolation kit (Roche), following manufacturer instructions. DNA was frozen to − 20 °C until molecular analysis. One-step real time polymerase chain reactions (qPCR) based on SYBR-Green dye and using primers and PCR conditions previously described by Forsgren and Fries (2010) [56].

### Expression of immune system genes

Genes involved in three inducible immune pathways in honey bees were studied: Toll, Janus kinase (JAK/STAT) and Imd [26]. Expression of the following genes was measured using specific primers: *defensin*-1 [57], *dorsal* [33], *domeless* [33] and *relish* [33]. Total RNA extracts obtained as described above were used to prepare cDNA with the PrimeScript RT Reagent Kit (Clontech, Takara). RNA extract (2 μl) was incubated with 2 μl of PrimeScript Buffer, 0.5 μl of PrimeScript RT enzyme, 0.5 μl of oligo(dT) primer, 0.5 μl of Random 6 and 4.5 μl of RNase/DNase-free water for 15 min at 37 °C and 5 s at 85 °C. The resulting mixtures were diluted 1:10 with molecular biology-grade water for a total of 100 μl cDNA template for quantitative PCR. All samples were analysed in parallel using a SYBR Fast Universal qPCR system (KAPA Biosystem) [54].

Individual reactions contained 5 μl of master mix (buffer and enzyme), 2 μl of cDNA template, 0.5 μl of forward and reverse target primers (5 μmol, l:1), and 2.2 μl of molecular biology-grade water. Reactions were cycled on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the following conditions: 5 min at 95 °C followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72° for 7 s, during which fluorescence measurements were taken. A final melt curve analysis was conducted at 95 °C for 15 s and 65 °C for 15 s. Each target gene was assayed for all samples on a single plate. A sample in which levels of all four viruses were undetectable was analysed to provide an uninfected reference as a default value in the Ct analysis.

Levels of expression of target genes were normalised to that of the β-actin gene. These normalised expression levels were compared among the seven colonies and between colonies positive or negative for each virus.

### Statistical analyses

Study variables are shown in Table 4. IAPV load and SBV load were not included in the final analysis, since all sample tested negative for these viruses. Differences in all variables were assessed for statistical significance using the non-parametric Mann-Whitney U test, since the data for all variables showed a skewed distribution. Virus load data were $\log_{10}$-transformed before statistical analyses, which were performed using SPSS version 22.0 [58]. $P < 0.05$ was considered significant.

**Table 4** Summary of study variables

| # | Variable | Type | n |
|---|---|---|---|
| 1 | Colony ID | Categorical | 7 |
| 2 | DWV load | Continuous | 57 |
| 3 | BQCV load | Continuous | 57 |
| 4 | *Varroa destructor* infestation rate | Continuous | 57 |
| 5 | *Relish* expression value | Continuous | 57 |
| 6 | *Defensin* expression value | Continuous | 57 |
| 7 | *Domeless* expression value | Continuous | 57 |
| 8 | *Dorsal* expression value | Continuous | 57 |
| 10 | Spring-Summer season | Categorical | 28 |
| 11 | Month | Categorical | 12 |

Colonies were also classified according to their DWV load, i.e. high, medium, low DWV load and free of virus according Amiri et al. (2015), in order to establish differences between immune gene level expressions in these groups.

Using multivariate Spearman correlation analysis, potential correlations among DWV, BQCV and *Varroa destructor* loads were explored across all seven colonies of the apiary over the 10 months of the study. Then, each colony was analyzed separately for these correlations. In the same way, Spearman correlations between pathogens and immune system gene expression were analyzed firstly across all colonies in the apiary and then for each colony. However, statistical analysis focused on individual colonies due to two reasons: 1) only three colonies survived for the entire period of study, which limited the analysis and 2) colonies are super organisms, at least in terms of their basic physiology, therefore individual conditions could be determinant for each colony. Immune system gene expression was also compared among colonies showing undetectable, low, medium or high virus load. Differences in study variables between collapsed and survived colonies were assessed for significance using the Mann-Whitney *U* test.

#### Abbreviations

BQCV: Black queen cell virus; DWV: Deformed wing virus; IAPV: Israeli acute paralysis virus; PAMPs: Pathogen Associated Molecular Patterns; PBS: Phosphate-Buffered Saline; PRRs: Pattern Recognition Receptors; qPCR: Quantitative Polymerase Chain Reactions; RNAi: Interference Ribonucleic Acid; RT-qPCR: Real-Time Reverse transcription-Polymerase Chain Reactions; SBV: Sacbrood bee virus

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#### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

SB, MVR and JSV conceived this research and designed experiments; FP participated in the design and interpretation of the data; SB and FMR

performed experiments and analysis; SB and MVR wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**
The study involved the European honey bee (*Apis mellifera*), which is neither an endangered nor a protected species.

**Consent for publication**
Not applicable

**Competing interests**
The authors declare that they have no competing interests.

## Publisher's Note



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# Chapter 3

## Study of the influence of the environment and nutrition on colony health

CHAPTER 3

# Objective 3

## Study of the influence of the environment and nutrition on colony health

**Subobjective 3.1** To determine the importance of pollen diversity intake in relation to viruses and *Varroa destructor*

**Subobjective 3.2** To determine the importance of apiary location in colony health

**Article submitted to peer reviewed journal**

- **S. Barroso-Arévalo,** M. Vicente-Rubiano, J.A. Ruíz, A. Bentabol, J.M. Sánchez-Vizcaíno. "Does pollen diversity influence honey bee colony health?" Submitted to Spanish Journal of Agriculture Research.

**Proceedings**

- **S. Barroso-Arévalo,** M. Vicente-Rubiano, J.A. Ruíz, A. Bentabol, J.M. Sánchez-Vizcaíno. "Relationship between landscape characteristics and colony health in *Apis mellifera* in Tenerife Island (Spain)". 9th Annual Meeting of EPIZONE (September 2016), Madrid (Spain). Oral communication.
- **S. Barroso-Arévalo,** M. Vicente-Rubiano, J.A. Ruíz, A. Bentabol, J. Goyache, J.M. Sánchez-Vizcaíno. "Variabilidad de polen y estado sanitario de las colmenas". IV Congreso Ibérico de Apicultura (April 2016), Salamanca (Spain). Oral communication.
- **S. Barroso-Arévalo,** M. Vicente-Rubiano, J.A. Ruíz, A. Bentabol, J. Goyache, J.M. Sánchez-Vizcaíno. "Variabilidad de polen y estado sanitario de las colmenas". I Jornadas de Investigación en Doctorado (VETINDOC) (June 2015), Madrid (Spain). Oral communication.

# Resumen

Los factores involucrados en las pérdidas en las colmenas de abejas *Apis mellifera* son numerosos, existiendo una gran controversia en relación a la importancia relativa de cada uno de ellos. Sin embargo, la nutrición y el medio ambiente han sido postulados como elementos claves para el funcionamiento adecuado de la colmena. Su papel parece ser determinante sobre todo en presencia de otros factores como la infección por el virus de las alas deformadas y el ácaro *Varroa destructor*. Asegurar la disponibilidad de polen y garantizar con ello la ingesta de todos los nutrientes necesarios podría contribuir a la mejora de la salud de las colmenas. Recientes estudios han apuntado, además, que la ingesta de polen más diverso podría tener un efecto positivo sobre las abejas. Todo ello se verá influenciado, a su vez, por la presencia y disponibilidad de los recursos, lo cual será determinado por la localización de los apiarios.

Para tratar de dar respuesta a estas cuestiones, en el presente capítulo de esta tesis doctoral se describe un estudio realizado en ocho colmenares de la isla de Tenerife que tuvo por objetivo evaluar la relación entre la salud de las colmenas y la diversidad del polen recolectado por las abejas, así como las condiciones medio ambientales de los apiarios. Se seleccionó la isla de Tenerife como modelo ya que cuenta con unas condiciones medio ambientales muy particulares, lo que conlleva la aparición de distintos micro climas. Para la realización de este trabajo, se recolectó el polen de tres colmenas por cada uno de los ocho colmenares incluidos en el estudio, en el periodo comprendido entre abril y septiembre de 2014, determinándose la biodiversidad de cada muestra. También se evaluó la carga de DWV, BQCV, IAPV, SBV y *Varroa destructor*, así como la fortaleza de las colmenas. Las condiciones medio ambientales de cada apiario fueron analizadas espacialmente, llevando a cabo una valoración de los emplazamientos en base a criterios descritos en la bibliografía.

La diversidad de polen no se correlacionó significativamente con la carga de DWV o la fortaleza de las colmenas, pero sí se correlacionó positivamente con los niveles de varroa, aunque el coeficiente fue muy débil. En contraste, la carga de DWV se correlacionó con la infestación por varroa y ambas variables se correlacionaron negativamente con la fortaleza de las colmenas. Además, la evaluación espacial de los colmenares evidenció que las colonias más débiles estaban localizadas en paisajes con áreas menos adecuadas para la producción apícola.

Estos resultados sugieren que el binomio DWV-varroa, así como las características del paisaje, influyen de manera significativa en la fortaleza de las colmenas, mientras que la diversidad de polen por sí sola no parece tener una relación directa con la salud de las colmenas, al menos con el diseño experimental propuesto. Así pues, los hallazgos del presente capítulo resaltan la utilidad de DWV y varroa como factores predictivos de pérdidas en las colmenas y sugieren la necesidad de evaluar cuidadosamente la ubicación de los colmenares para garantizar los recursos nutricionales y la viabilidad de las colmenas.

# Abstract

Colony losses of the western honey bee *Apis mellifera* have increased alarmingly in recent years. These losses have been attributed to nutritional deficiency, environmental conditions, viral infection and the global presence of the ectoparasite mite *Varroa destructor*. The lack of nutritional resources seems to be one of the main threats to honey bee colonies, as well as deformed wing virus (DWV) and varroa complex. Ensuring pollen availability may improve colony health, so the present study aimed to examine whether the diversity of pollen collected by the colony as well as landscape characteristics of apiaries influence colony health.

Colonies at eight apiaries on Tenerife Island (Canary Islands, Spain) were sampled in late summer to determine colony strength, presence of varroa and load of DWV. Pollen was collected during six months and analyzed. Landscape of each apiary was spatially analyzed. Pollen diversity did not correlate significantly with colony strength or the load of DWV, but it positively correlated with varroa levels. In contrast, DWV load correlated with varroa infestation, and both variables negatively correlated with colony strength. Weak colonies were located in landscapes with areas less suitable for bee nutrition.

These results suggest that DWV and varroa infection as well as landscape characteristics influence colony survival, while pollen diversity on its own does not seem to have direct relationship. Our findings highlight the usefulness of DWV and varroa as predictors of colony losses, and they suggest the need to carefully assess honey bee apiary location in order to ensure adequate nutritional resources.

# 3.1 Introduction

Growth and survival of honey bee colonies are strongly related to the availability of food resources and the suitability of land uses, climate, temperature and other factors for ensuring adequate nutrition (Brodschneider & Crailsheim, 2010; Keller, Fluri, & Imdorf, 2005). Malnutrition, together with starvation, is considered one of the leading causes for declining bee populations (Naug, 2009; Stanley, 1974), since nutritional stress can weaken the immune system, whose maintenance is costly (Field et al., 2002). It may also contribute to the alarming losses of honey bee colonies around the world in recent years, which likely reflect a complex mixture of factors including malnutrition, environmental conditions, intensive beekeeping (Antoine Jacques et al., 2017), infection with viruses such as deformed wing virus (DWV) and the global presence of the ectoparasite mite *Varroa destructor* (vanEngelsdorp et al., 2009).

Honey bees obtain nutrients from nectar, which is mainly carbohydrates, and pollen, which provides proteins, amino acids, lipids, starch, sterols, vitamins and minerals (Roulston & Buchmann, 2000; Stanley, 1974). Pollen provides protein, which are essential for development of hypopharyngeal glands in adult worker bees and for production of royal jelly to support normal growth, reproduction function and brood rearing (Haydak, 1970; Standifer, 1980). Pollen is also important for the physiological metabolism (Alaux et al., 2011), optimal development of the immune response (Alaux et al., 2010) and resistance to pathogens and pesticides (vanEngelsdorp, Hayes, et al., 2009). Therefore, lack of pollen results in poor physiological conditions and increased susceptibility to external threats at individual level, which can lead to into colony losses (Keller et al., 2005). Therefore, to study the relationship between colony pollen collection and health would allow better knowledge of the role of this important factor involved in the current loss of honey bee populations.

While most studies have focused on whether the quantity of pollen intake influences honey bee health and resistance to disease, if these outcomes are also influenced by the quality, diversity or richness of pollen is unclear. The composition or relative amounts of amino acids in pollen differ across floral species, raising the question of whether these differences play a crucial role in maintaining an optimal immune response of protective effects by pollen (Di Pasquale et al., 2013). Previous studies have revealed that a diet of diverse (polyfloral) pollens may lead to greater immune competence than a monofloral diet (Alaux et al., 2010). This poses the question of whether insufficient pollen diversity can compromise the honey bee immune system (vanEngelsdorp et al., 2009) and thereby render colonies more susceptible to pathogens that have been associated with colony losses around the world (Cornman et al., 2012; Cox-Foster et al., 2007; Kang et al., 2015). Therefore, the present study aimed to examine the possible effects of pollen diversity on honey bee colony health.

In addition to pollen diversity, the dissemination of pathogens in the apiary can strongly influence colony health (Carreck et al., 2010; Dainat et al., 2012c). Among pathogens related to colony losses, honey bee viruses are widespread and most of them cause covert, persistent infections (Evans & Hung, 2000), making them difficult to detect and eradicate. Environmental factors, nutritional deficiency or parasite infestation can lead to a stress status in the colony that promotes viral replication and makes the colony less able to resist viral infection triggering the appearance of clinical symptoms (Dainat et al., 2012; Dainat & Neumann, 2013). Some viruses are particularly important due to their distribution and high prevalence, such as black queen cell virus (BQCV) and sacbrood bee virus (SBV), and/or their virulence, such as Israeli acute paralysis virus (IAPV) and deformed wing virus (DWV). Remarkably, the combined presence of deformed wing virus (DWV) and the widespread *Varroa destructor* mite correlate with colony collapse and have been proposed as colony survival markers (Carreck et al., 2010; Francis, Nielsen, & Kryger, 2013; Kielmanowicz et al., 2015; Nazzi & Pennacchio, 2018).

Food quantity and diet composition have been shown to influence honey bee physiological and immunological functions (Di Pasquale et al., 2013). Consequently, the capacity to face infectious diseases and especially viral covert infections could potentially be affected by diet. Elucidating how pollen diversity influences the infection of bee viruses would help clarify the role of diet on colony health. Since nutritional deficiency can trigger this stress, we hypothesized that colonies with poor pollen diversity may be less healthy and, in the presence of DWV and/or varroa, more likely to suffer damage due to these pathogens. To test this hypothesis, we studied whether variations in pollen diversity in the diet influence the dynamics of four bee viruses and the appearance of viral symptoms in the colonies. Due to its importance as vector of viruses, dynamic of the *Varroa destructor* mite was also considered for this objective. A secondary objective was to identify geographical and climatic factors from apiary locations that are related to increase presence of viral symptoms, depopulation or high viral and/or varroa loads thus having a negative effect in colony health.

# 3.2 Material and methods

## 3.2.1 Study area

The island of Tenerife (Canary Islands, Spain) was selected to perform the study for three main reasons. First, it is a limited area that has high beekeeping activity (5% of Spanish hive census, MAGRAMA, 2015). Second, it offers very different environmental conditions for apiary location attending to differences in altitude and microclimates. Third, it has a diverse and characterised subtropical flora usable by honey bees (Henríquez Jiménez & Paricio Núñez, 1979). Specifically, nearly 100 of approximately 740 endemisms present in the island can be used by honey bees. The specific location of each apiary is shown in Figure 1.

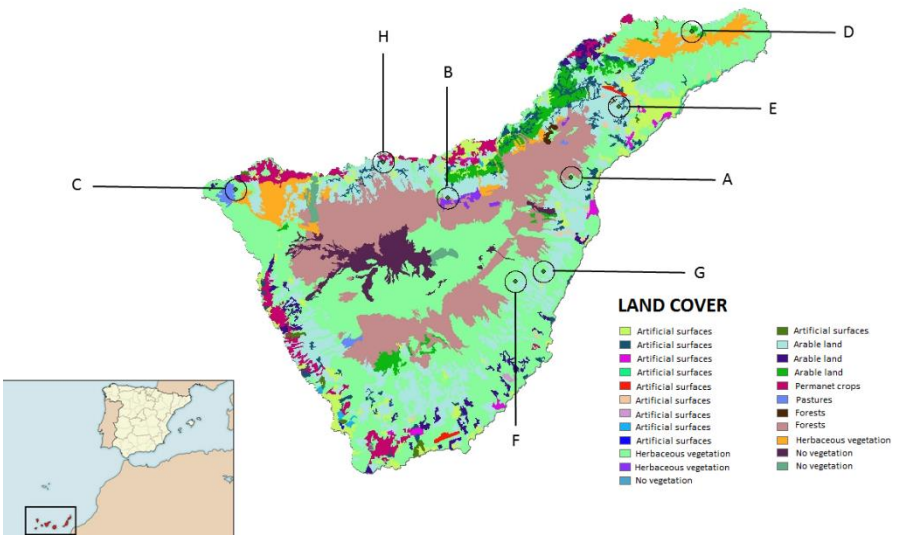


**Figure 1.** Location of apiaries in the study area.

## 3.2.2 Sampling design

We studied eight apiaries of *A. mellifera mellifera* (denominated A-H), all of which were maintained in natural environments. If there were crops, those were small family crops (Figure 1), which are a common type of crop in Tenerife Island. They are small-size orchards of horticultural crops and fruit trees, in which the use of pesticides tend to be avoided. The apiaries had a size between 20 and 40 Langstroth hives, as is common in Tenerife´s beekeeping. At each apiary, three colonies were selected and sampled for honey bees and pollen, thus 24 colonies were included in the study. At each apiary, colonies selected were re-queened with queens of the same age and genetic characteristics, and standardized to have 6-7 frames covered with bees and 2-3 frames covered with capped brood, in order to select strong colonies. Pollen was collected during the spring and summer, from April to September of 2014, since rainfall, temperatures and flowering vary between these two seasons (Henríquez Jiménez & Paricio Núñez, 1979). Seven pollen collections were performed in apiaries A and E and nine collections in apiaries B, C, D, F, G and H. Trial scheme is shown in Figure 2. Pollen pellets were collected from pollen traps that were closed

24 h before sampling at that were placed at the hive entrance. All pollen samples were collected at the same day of the month in all the studied colonies.

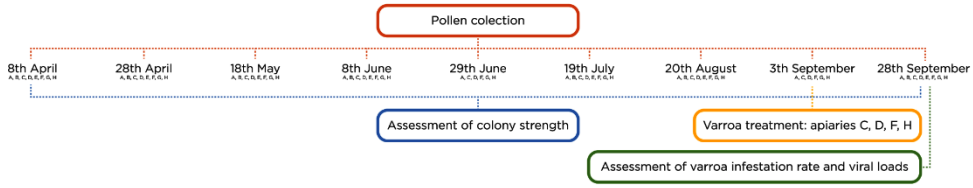


**Figure 2.** Schematic illustration of trial schedule and map. 3 colonies in each site were requeened and equalized to have 6-7 bee frames and 2-3 brood frames. Colonies were sampled two times during the trial for hive strength, and one for bee viruses, and *Varroa destructor* counts. Pollen was collected nine times (apiaries A, C, D, F, G, H) and seven times (apiaries B and E).

Colony strength was measured at the start (April 2014) and at the end of the study (September 2014) by the Veterinary Technician. At the end of the pollen study (September 2014), two type of honey bee samples were collected from each colony (adults and brood). A total of 48 samples (24 adult samples and 24 brood samples) were collected in sterile containers, were keep on dry ice for the transport and frozen at -80º C until laboratory analyses. Adult samples consisted of approximately 50 adult bees that were carefully taken by hand from the hive entrance or the honey combs of each colony. Additionally, one portion of brood comb was taken from each colony. These samples were analysed to detect the presence of four bee viruses (DWV, BQCV, IAPV and SBV). Given that beekeepers selected strong colonies, we assumed that the initial state was homogeneous in all the apiaries and, therefore, we considered that at least one pathogen sampling could provide useful information about the evolution of the colonies in regard with pollen collection analysis. . Regarding colony treatments, apiaries C, D, F and H were treated against *Varroa destructor* during the month before the honey bee sampling.

### 3.2.3 Assessment of bee population and symptoms

The honey bee population in each colony at the time of sampling was estimated by a veterinarian technician, who took into account the number of honey bee´s and brood´s combs (Delaplane et al., 2013b). Colonies with signs of poor population (low activity in the entrance of the colony or fewer than five bee and brood combs were categorized as having a "poor population"; otherwise, colonies were categorized as having an "adequate population", considering the beekeeping managing and the time of the year. Health-related events were also observed in the sampled colonies, such as viral symptoms (deformity in wings and nervous symptoms), abnormal presence of *Varroa destructor* and mortality.

The presence of any of the health events already mentioned was considered together with the assessment of bee population in each colony. Colonies that showed at least one symptom and/or were classified as having a "poor population" were classified as "weak colonies", whereas colonies with an "adequate population" and without any symptoms were classified as "healthy colonies".

### 3.2.4 Assessment of mite infestation levels

Mite infestation levels on adult honey bees were determined at bee sampling (on 28 September 2014). Mite infestation levels were calculated as the percentage of mites per 100 worker honey bees in the sampled colonies, using the soapy water method described in "Standard methods for varroa research" in the COLOSS BEEBOOK (Dietemann et al., 2012). Briefly, 300 adult bees were collected from the colony from the sides of the unsealed brood combs, shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. The percentage of mites was calculated as follows:

$$\% \text{ infestation} = (\text{no. mites} / \text{no. bees counted}) \times 100\%$$

### 3.2.5 Detection and quantification of bee viruses

For each colony, separate pools of 10 honey bees and 10 pupae were crushed in a mortar in 5 mL of sterile phosphate-buffered saline (PBS) (Dietemann, D Ellis, & Neumann, 2013). This number of honey bees was used as we encountered difficulties in collecting pupae in some colonies. However, it allows detection of well-established viral infection in the colony as detection is performed with 95% probability of detection if at least 25% of honey bees are infected (Dietemann et al., 2013). This approach of detecting infections at an incidence of at least 25% is useful for *A. mellifera* studies because low viral load usually does not have a major impact on the colony for the viruses under study. In total, two homogenates were obtained from each colony. These homogenates were stored at -80 ◦C.

RNA was extracted from honey bees´s and pupae´s homogenates using the column-based Nucleospin II Virus® kit (Macherey Nagel) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and maintained at −20ºC until analysis. To ensure the correct preservation of RNA, RNA samples were analysed by RT-PCR during the days following the extraction. After PCR analysis, samples were stored at -80º.

Samples were tested for presence and load of the following four bee viruses in separate one-step real-time reverse transcription-polymerase chain reactions (RT-qPCR) based on SYBR-Green detection as described: DWV (Kukielka et al., 2008), BQCV (Kukielka et al., 2008), IAPV (Maori et al., 2009) and SBV (Amiri et al., 2015). Viral load in positive samples was quantified absolutely using a standard curve constructed with serial 10-fold dilutions of known amounts of plasmid DNA, starting from cloned fragment of these virus (from DWV and BQCV) into a PGemT® TA cloning vector (Promega) in accordance with the manufacturer's instructions. The slope and intercept of each calibration curve were determined from a best-fit line

with a correlation coefficient of 0.99. Viral loads were expressed in genome equivalent copies (GEC) per µl of homogenized bee sample and assigned to one of four categories of infection (Amiri et al., 2015): virus-free (GEC=0); low virus titer, $0<GEC<10^3$; medium virus titer, $10^3 \leq GEC<10^7$; and high virus titer, $GEC \geq 10^7$. Plasmid confirmed as positive to each virus by PCR were used as a positive control for DWV, BQCV, SBV and IAPV.

### 3.2.6 Determination of pollen diversity

A pollen trap was placed at the hive entrance to collect pollen brought by foragers returning to the hive. Pollen was collected at 24 h after the trap had been closed, then sent by beekeepers to a central storage facility at the "Casa de la Miel" (Cabildo, Tenerife).

Pollen species and richness were established though palynological tests based on the method of Louveaux et al. (Louveaux, Maurizio, & Vorwohl, 1978) with some variations. Briefly, pollen was diluted in distilled water, stained with glycerogelatin with basic fuchsine, and then examined under a microscope to determine species. Protein content was determined in quintuplicate using Kjeldahl analysis. As a measure of diversity, we calculated the Shannon index, according to the following equation (Ramírez-Arriaga, Navarro-Calvo, & Díaz-Carbajal, 2011; Shannon & Weaver, 1949):

$$H = -\sum_{i}^{n} pi \ln pi$$

Where H: Shannon-Weaver diversity index, pi: proportion of each pollen type i encountered in the sample (counted in 500 grains of pollen per sample), ln: natural logarithm.

### 3.2.7 Climatic and environmental parameters

Climatic and environmental conditions were studied for an area of radius 1,500 m around each apiary based on geographic coordinate system (GPS). This area is an effective foraging area of honey bees (Winfree, Williams, Dushoff, & Kremen, 2007). Land uses, forest cover, anthropogenic infrastructure, and landscape configuration and composition were obtained from Corine Land Cover ("Corine Land Cover, seamless vector data (Version 17)," 2006) and were represented geographically using GIS tools (ESRI, 2011). Temperature and precipitation data for Tenerife were obtained from the Weather Underground website ("Weather Underground, IBM," 1993).

Habitat quality was measured in terms of 5 factors: number of land cover types (F1), number of important vegetable species for bees (F2), distance to permanent watercourses (F3), harvestable area unfragmented by infrastructures (F4), presence of animal farms near the study area (F5), and crop surface (F6). These factors were defined based on a literature review (Asensio et al., 2016; Decourtye, Mader, & Desneux, 2010; Gallant, Euliss, & Browning, 2014; Naug, 2009; Simioni et al., 2015) and the forestry map of the Canarias Government (Grafcan, 2015).

F1 was considered good when there were more than 5 land cover types. F2 was identified through an extensive literature research (Kunkel, 1991) and using the forestry map, and protein composition of each species was used in order to classify them; the presence of more than 10 vegetable species for bees was considered the best condition for the study area. The absence of major roads, urban areas, and large bodies (coast) of water or industries around the study area was considered the best condition for F3. Closeness to rivers and streams (≤ 50 m) was considered the best condition for F4. The absence of animal farms near the study area was considered the best option for F5, as well as the absence of crop surface (F6).

### 3.2.8 Statistical analysis

Statistical analyses were performed using SPSS 22 (IBM, 2013). All data were stratified into 8 variables per colony and type of sample: 3 categorical (Apiary –A to H-, Type -adult or brood-, Health status –healthy or weak-) and 6 continuous (DWV, BQCV, varroa, pollen richness, pollen diversity and viral prevalence). Data for continuous variables were re-scaled into logarithmic scale.

Pathogen load was measured separately in adults and brood for each colony, and the higher load value was taken to be the value for the colony for the given pathogen. Pairwise comparisons in pathogen load between brood and adult samples were used, as well as between "weak" and "healthy", using the non-parametric Mann-Whitney *U* test (with a threshold of p-value < 0.05). Potential relationships among pathogen loads and between pathogens and pollen richness were explored using Spearman's rank correlation.

A possible relationship between pollen richness and colony health category (weak, healthy) was explored using binomial logistic regression. Colony strength category was the dependent variable and was dichotomized as 1="weak colony" or 0="healthy colony".

## 3.3 Results

### 3.3.1 Viral load per colony and per sample

At the sample level (adult and brood samples), DWV was present in 42 of 48 samples; load was high in four samples, medium in 11, low in 24 and 5 samples were free of DWV. BQCV was detected in 35 of 48 samples; load was medium in 11 samples, low in 24 and 13 were free of virus. At the colony level, DWV was detected in 22 of 24 colonies (91.66%), while BQCV was detected in 20 of 24 colonies (83.33%). SBV and IAPV were not detected. Mean viral load was $<10^6$ GEC/µl (medium and low load) in 41 of 48 samples in the case of DWV, and in 45 of 48 samples in the case of BQCV.

At apiary level, only one of eight apiaries (apiary B) showed high DWV load, while no colonies showed high BQCV load (Figure 3). The load for both viruses was medium in the majority of cases (28 of 48 samples), especially in apiaries A, D and G, which were highly infested by varroa and had significantly higher DWV load (Mann Whitney U test, P=0.002). DWV load was lower in apiaries treated against varroa (Mann Whitney U test, P=0.001). DWV loads were significantly higher in brood samples with a varroa infestation level higher than 15% (Mann Whitney *U* test, P=0.005).

BQCV loads were significantly higher in adult samples than in brood samples (Mann Whitney U test, P=0.008). The results of DWV and BQCV load in adult and brood samples are summarized in Figure 3. Therefore, colonies with highest BQCV load were likely to have fewer combs of brood (Mann Whitney *U* test, P=0.004).

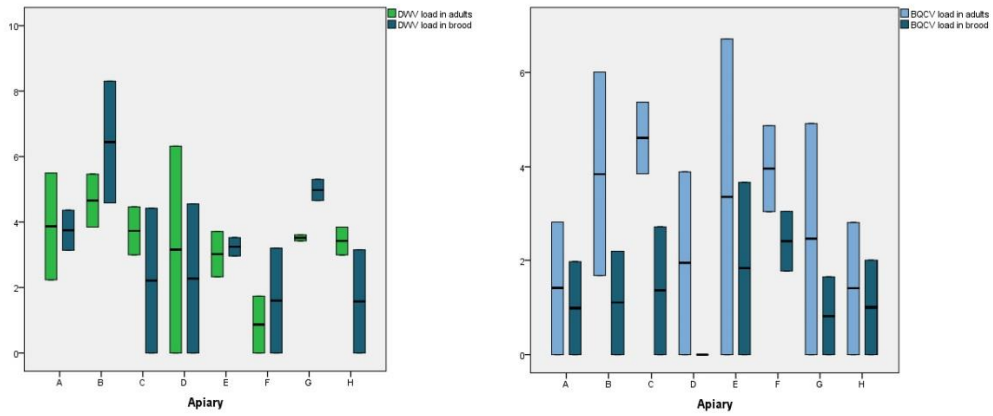


**Figure 3.** Log-transformed mean loads of DWV and BQCV (GEC/µl) in adult and brood samples at apiaries A to H.

### 3.3.2 *Varroa destructor* prevalence

Varroa was detected in 18 colonies (90%) at highly variable infestation levels from 0.35% to 42%; 11 of 18 positive colonies showed infestation levels <10%. Apiary B showed the highest infestation level, where mites were observed at this

apiary during inspection, and the three colonies showed clear symptoms of infestation. Mite load correlated positively with DWV infection intensity (Spearman R=0.504, P=0.002). Apiaries treated against varroa showed significantly lower mite infestation levels than untreated apiaries (Mann Whitney U test, P<0.001).

### 3.3.3 Correlation between viral load, varroa infestation rate and pollen diversity

In order to identify any potential relationships between viral load, varroa infestation rate and pollen richness and diversity, a spearman correlation analysis was performed. DWV load and varroa infestation rate showed significant correlation, as well as varroa infestation rate and pollen diversity, and pollen diversity and pollen richness. Results are shown in Table 1.

**Table 1.** Spearman correlation analysis to identify relationships between pathogen load and pollen diversity. DWV: Deformed Wing Virus; BQCV: Black Queen Cell Virus. Significant results are highlighted in bold.

| Parameter | Correlation coefficient | p value | N |
|---|---|---|---|
| DWV-BQCV | 0.256 | 0.067 | 48 |
| **DWV-Varroa** | **0.504** | **0.002** | **48** |
| BQCV-Varroa | -0.230 | 0.101 | 48 |
| DWV-Pollen diversity | 0.147 | 0.317 | 48 |
| BQCV-Pollen diversity | 0.189 | 0.594 | 48 |
| **Varroa-Pollen diversity** | **0.384** | **0.012** | **48** |
| Pollen richness-Pollen diversity | 0.771 | >0.001 | 68 |

### 3.3.4 Honey bee population and symptomatology

Six of 24 colonies were classified as “weak colonies”. These colonies belonged to apiaries B, D, E, F, G and H. The most frequently observed symptom was presence of wing deformities, followed by visual detection of varroa in the colony. Compared to “healthy” colonies, “weak” colonies showed significantly higher DWV load (odds ratio 1.665, P=0.015) and varroa infestation rate (odds ratio 1.059, P=0.044), based on binomial logistic regression.

### 3.3.5 Pollen diversity

72 plant species were identified in pollen samples, with the most frequent species indicated in Table 2 together with associated protein content.

**Table 2.** Most frequent plant species identified through pollen analysis, and the corresponding protein content.

| Botanical specie | Protein % |
|---|---|
| ***Aspalthium bituminosum*** | 25.59 |
| ***Calendula arvensis*** | 16.32 |
| ***Carlina salicifolia*** | 16.7 |
| ***Castanea sativa*** | 25.99 |
| ***Cistus monspeliensis*** | 14.74 |
| ***Cistus symphytifolius*** | 16.59 |
| ***Citrus sinensis*** | 14.74 |
| ***Convolvulus floridus*** | 18.87 |
| ***Daphne gnidium*** | 13.72 |
| ***Echium plantagineum*** | 35.34 |

| | |
|---|---|
| ***Ilex canariensis.*** | 22.48 |
| ***Origanum vulgare*** | 14.72 |
| ***Rubus ulmifolius*** | 24.38 |
| ***Rumex sp.*** | 15.47 |
| ***T. Brassica*** | 20.88 |
| ***T. chamaecytisus proliferum*** | 25.72 |
| ***T. lilium*** | 17.1 |
| ***T. galactites tomentosa*** | 19.75 |
| ***T. papaver rhoeas.*** | 23.28 |
| ***T. papaver somniferum*** | 23.95 |

Availability of pollen from diverse botanical origins (estimated through the Shannon index) varied along the period of study. Pollen richness varied throughout the period of study, ranging from 2 to 16; it was significantly higher in spring (April to June) than in summer (June to September) (Mann Whitney U test, P=0.023). Shannon index ranged from 0.25–2.78 and no differences between seasons were detected (Figure 4). Comparison of pollen richness and pollen diversity (Shannon index) in the last pollen collection (coinciding with pathogen assessment) is shown in Figure 5.

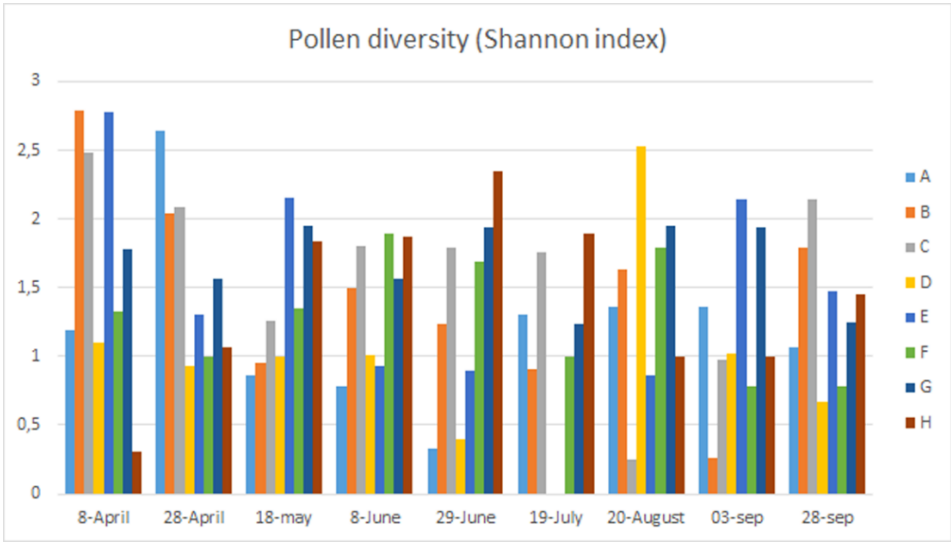


**Figure 4.** Seasonal variations of pollen diversity (Shannon index) per apiary.

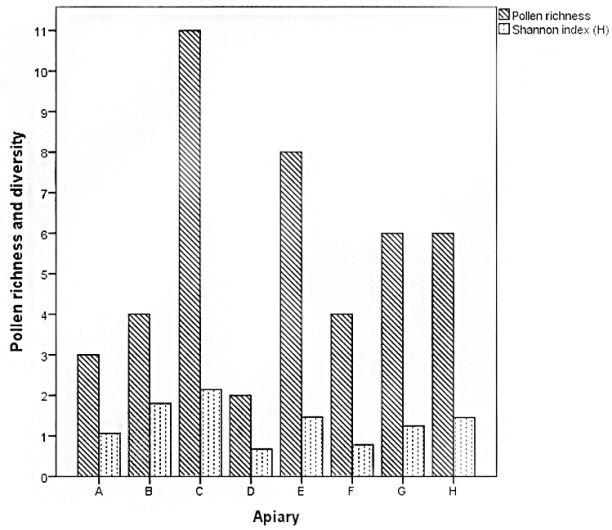


**Figure 5.** Comparison of pollen richness and diversity (Shanon index) in the last pollen collection (28 September), coinciding with the assessment of varroa infestation rate and viral loads.

Botanical species identified also varied along the study. In September, before honey bee sampling, the most frequent plant species were *Cistus monspeliensis, Kleinia neriifolia, Castanea sativa, Rubus ulmifolius* and *Galactites tomentosa*. These

plants are considered flora of bee interest (Silva & Restrepo, 2012). *C. monspeliensis* has high pollen content although relatively low protein content. *K. neriifolia,* which is endemic in Tenerife, is often used by honey bees, and it produces a large amount of pollen (Kunkel, 1991). *C. sativa* provides nectar and honeydew, its pollen has a high protein content (25.99%) and it is frequently the only food supply for honey bees. *R. ulmifolius* is a source of nectar and pollen, especially when flowers bloom in summer, and it has high protein content. In contrast, *G. tomentosa* supplies nectar but relatively little pollen (Rodriguez-Franco & Cuevas, 2013).

### 3.3.4 Environmental parameters

Five of eight apiaries were located 500 meters above sea level, while the other three were below that altitude. Different climatic areas were found at different altitudes: coastal zone was under 300 meters; middle area was at 300-600 meters; and alpine area was above 600 meters. Apiaries C and H were located in the coastal zone, where precipitation is low and temperature high. Apiaries A, B and G were located in the alpine area, which registers the most rainfall on the entire island as well as low temperatures (Rodriguez-Franco & Cuevas, 2013). Varroa infestation was significantly higher in apiaries located 600 meters above sea level (Mann Whitney *U* test, $P<0.001$).

Habitat conditions were worse (based on the 8 factors considered) around apiaries that were classified as "weak" colonies (i.e. dead honey bees around the colony, poor population) and that showed higher pathogen and varroa levels per apiary. These less favourable habitat conditions included the presence of urban areas, roads, abandoned fragmented bee habitat and reduced bee foraging area, all of which are associated with lower availability of natural resources. For example, more than one virus infected all colonies in apiary B, located near cattle pasture and the coast, factors that fragment the habitat; 2 of 3 colonies in this apiary had high DWV load, and one colony showed a varroa infestation level of 42%. In addition, the foraging area of this apiary contained two goat farms and one pig farm. Apiary D,

which was located very close to the coast and had much of its foraging surface occupied by family crops, showed high DWV load and medium BQCV load, and it had weak colonies that showed symptoms (deformed wings) and poor population. In apiary E, dead honey bees lay around one colony and other symptoms related to varroa and DWV presence were observed (deformed wings). The foraging area contained crops, urban areas and cattle pasture, as well as 14 farms of pigs, cattle and goats (Grafcan, 2015). All these characteristics can fragment habitat, reducing the landscape heterogeneity and availability of food resources for honey bees.

# 3.4 Discussion

Malnutrition is one of the causes of honey bee losses, as it origins poor vigorous population and brood failure. Rarely is pollen entirely lacking from natural environments inhabited by honey bees, so the malnutrition that can drive colony losses is more a problem of poor pollen quality and diversity, and the latter has been linked to nutritional quality (Alaux et al., 2010). Nutritional quality has been linked to adequate colony development (Brodschneider & Crailsheim, 2010). Therefore, we investigated whether pollen diversity accessible to honey bees at eight commercial apiaries on Tenerife Island (Spain) significantly affected colony health, which we measured in terms of appearance of symptoms, honey bee population, load of four honey bee viruses and varroa mite levels. Our results suggest that pollen diversity does not substantially influence colony health. Although varroa levels significantly correlated with pollen diversity, Spearman´s coefficient was weak (0.384). While these findings may reflect the fact that possible correlations between pollen diversity and health parameters were tested only once (at the end of the summer), they may also indicate the need to focus on other factors that are more likely to play a strong role in determining honey bee health.

In our study, pollen diversity was higher in April; it fell at the beginning of the summer (end of June and July) and thereafter did not follow any clear pattern

except that it remained lower than in the spring in almost all the apiaries. These results are consistent with the decline in bloom on the Canary Islands during the summer (Henríquez Jiménez & Paricio Núñez, 1979). Brood blocking and death of adult honey bees in this season contribute to lower foraging activity and therefore smaller colony population. Despite the higher pollen diversity in spring, and the association between higher diversity and protein content, we did not observe the positive correlation between pollen diversity and resistance to disease reported by Antunez et al. (Antunez et al., 2015). On the contrary, we found that higher pollen diversity correlated with higher varroa levels. A possible explanation for this might be that a more diverse diet may have a positive impact on individual and social immunocompetence and physiology, as suggested in previous works (Alaux et al., 2011; Alaux et al., 2010; Di Pasquale et al., 2013). As a consequence, it may be reflected in an increase of brood production, which would encourage mite reproduction (Evans & Cook, 2018). Further studies on this topic needs to be undertaken before the association between high pollen diversity and varroa levels is more clearly understood. Thus, these results suggest that at the apiaries in our study, and under the particular conditions of beekeeping in Tenerife, diverse diet by itself may not directly benefit colony health. Instead, other factors working alone or in conjunction with pollen diversity may influence colony resistance to disease.

Viruses can seriously affect colony survival (McMenamin et al., 2018). They usually persist in colonies as covert infections, without causing obvious damage to honey bees. Under certain circumstances, viruses start replicating and infections can become overt and show symptomatology. Adverse environmental conditions (i.e., habitat, climatology), the varroa pressure and changes in the population structure of the colony might influence overwintering abilities of honey bees. In this scenario, opportunistic viral infections can promote and cause the decrease the survival ability of the colony. Therefore it was examined the load of four honey bee viruses in the apiaries of our study. Only two viruses were detected in the colonies: DWV was found

in 91.66% of colonies, while BQCV was found in 83.33%. Most colonies had only low or medium load of these viruses, which is lower than the load measured at apiaries on the Spanish peninsula (Asensio et al., 2016; Kukielka et al., 2008).Viral analysis was performed for adults and brood separately in order to detect potentially differential effects on the two populations. Only BQCV presented differences for the two populations, in which viral load was significantly higher in adults. This finding may be related to the viral ability to promote covert infections in the case of low pathogenic-viruses, being able to achieve high load in adults without causing mortality or any symptoms.

Both varroa infestation and DWV infection strongly influenced the health status of the apiaries in our study. Weak colonies, which showed symptoms or poor population, also had the highest levels of DWV and varroa. None of the colonies died during the study, and therefore no relationship between pathogens and colony collapse was found. However, this is consistent with previous studies that associated DWV load with colony weakness, suggesting that it may be a good predictor of colony weakness and losses, as it was reported by some studies (Cornman et al., 2012; Cox-Foster et al., 2007; Kang et al., 2015; Kielmanowicz et al., 2015). Correlation between DWV and varroa loads was found, echoing previous studies (Cornman et al., 2012) and reflecting the fact that the mite effectively transmits viruses such as DWV (Ryabov et al., 2014). By feeding on hemolymph, varroa can directly injure honey bees while at the same time transmitting viruses, activating viral replication and suppressing the immune system, rendering the honey bees more vulnerable to disease (Yang & Cox-Foster, 2005). This can give rise to synergism involving virus-parasite-host immunity (Di Prisco et al., 2016), which can strongly alter the expression of several immune genes. These considerations suggest that DWV and varroa can act together to affect colony health, and that these negative effects are likely to be greater when brood is growing, since varroa is reproducing and thereby helping DWV to replicate. Further study is needed to clarify the roles of each

pathogen in the development of colony weakness. Future work should also examine whether loads of these and other pathogens interact with pollen richness, which negatively correlated with infection intensities of some viruses in previous work (Alaux et al., 2011) but not in the present study.

Mite control could help avoid colony losses associated with varroa and varroa-transmitted viruses, as well as reduce risk of strong DWV infection in brood. Anti-varroa treatment of apiaries C, D, F, and H considerably reduced DWV load in colonies, indicating that this virus is not efficiently transmitted horizontally in the absence of mites (Kang et al., 2015), and that vertical transmission routes are unlikely to lead to heavy loads in bee brood. Taking together, varroa infestation and DWV infection play a crucial role in the colonies. Monitoring viral load, especially DWV load, may help detect poor honey bee colony health and predict colony losses during overwintering.

In addition to pathogens, habitat conditions at the apiaries in our study strongly affected colony development: apiaries located near the coast showed worse population or symptomatology than other apiaries, and higher altitude was associated with lower colony strength. The diversity of habitat conditions across the apiaries provided a good opportunity for us to assess landscape effects. These findings emphasize the need for further research into effects of environment on honey bee colonies, and the need to take environment into account when locating hives. Landscape features that reduce resource availability, such as proximity to pastures or urban areas and land fragmentation may increase risk of colony loss and risk of collapse during overwintering (Asensio et al., 2016; Highfield et al., 2009; Schroeder & Martin, 2012). Therefore, future studies should deepen the study of environmental effect in honeybee colonies.

While the findings of this study should be treated with caution because of the limited sampling and pathogen analysis, these results nevertheless provide

evidence that pollen richness does not directly benefit honey bee colony health by itself. This may reflect the complex relationships among diet, breeding and pathogens: for example, colonies with access to abundant nutrients may breed rapidly, which at the same time allows rapid proliferation of varroa and the viruses that it transmits. In this way, changes in pollen diversity may exert effects at the colony level without affecting health markers such as pathogen load. Another possible explanation for our observed lack of correlation between pollen diversity and colony health is that a key determinant is not so much the species diversity in available pollen, but the composition of specific amino acids in that pollen. Our analysis of overall protein content in the different pollens sampled suggests that protein supply did not vary substantially along the study period, but it is still possible that the availability of critical amino acids did vary, which in turn may have affected colony resistance to disease. A third possible explanation for the lack of correlation between pollen diversity and health markers is that a key determinant is the immune strength of the colony (Alaux et al., 2010; Highfield et al., 2009), which we did not analyze directly. Future work should examine whether changes in the intake of specific amino acids affect health markers, and whether this diet factor or even pollen diversity directly affect the immunocompetence of individuals and the colony as a whole.

# 3.5 Conclusions

This study suggests that pollen diversity in Tenerife Island does not significantly affect honey bee colony health or resistance to pathogens by itself, at least based on the analysis of data collected at the end of the summer. Instead, varroa parasitism and the presence of DWV exerted the greatest stress on the colony at this time. This suggests that these two factors should be taken into account for assessing colony health.

# Chapter 4

## Analysis of the relationship among viruses and the *Varroa destructor* mite with colony vigour in Southern Spain

# Objective 4

## Analysis of the relationship among viruses and the *Varroa destructor* mite with colony vigour in Southern Spain

**Article published in a peer reviewed journal**

- **S. Barroso-Arévalo,** E. Fernández-Carrión, J. Goyache, F. Puerta, F. Molero, J.M. Sánchez-Vizcaíno. "High load of deformed wing virus and *Varroa detructor* infestation are related to weakness of honey bee colonies in southern Spain". Published in Frontiers in Microbiology. DOI:10.3389/fmicb.2019.01331

**Proceedings**

- **S. Barroso-Arévalo,** J. Goyache, J.M. Sánchez-Vizcaíno. "Análisis molecular de los principales patógenos en *Apis mellifera* y su importancia en el desencadenamiento del colapso en las colmenas". In I Jornadas de Investigación en Doctorado (VETINDOC), Universidad Complutense de Madrid. Facultad de Veterinaria, Universidad Complutense de Madrid (June 2015). Oral communication.

# Resumen

Como ha sido descrito en los capítulos anteriores, multitud de factores contribuyen en mayor o menor medida a las pérdidas continuadas en las poblaciones de abejas. Los apicultores reportan, además de un aumento de la mortalidad invernal de las colmenas, descensos en el vigor y la producción. La importancia de DWV y el ácaro *Varroa destructor* sobre dichas pérdidas ha sido ampliamente descrita en los trabajos previos. Sin embargo, comprobar la validez de estos hallazgos en un periodo de tiempo más prolongado, así como evaluar la capacidad de supervivencia de la colmena en presencia de estos dos patógenos, podría aportar valiosa información que permita frenar estas pérdidas. En el presente capítulo de esta tesis doctoral se analiza, además, la eficacia de la determinación del vigor de las colmenas por el técnico apícola como método para estimar la fortaleza de la colmena, en comparación con el método estandarizado (Delaplane et al., 2013b), con el objetivo de aportar nuevas herramientas al apicultor y a los investigadores.

Para este trabajo, se analizó la carga de DWV, BQCV, IAPV y SBV, así como la infestación por el ácaro *Varroa destructor* en las diez colmenas del colmenar experimental de la Universidad de Córdoba de manera mensual durante 21 meses. Además, se evaluó la fortaleza de las colmenas aplicando el método subjetivo estandarizado y mediante la determinación del vigor por parte del técnico apícola, procedimiento menos invasivo y más rápido con respecto al estandarizado.

La determinación del vigor como medida de fortaleza de la colmena mostró resultados similares a los del método estandarizado, presentando ventajas tales como una mayor rapidez y ser menos invasivo para la colmena. La carga de DWV y varroa se correlacionó negativamente con el vigor de las colmenas, siendo ambos patógenos identificados como los principales agentes involucrados en la debilidad de las colmenas mediante un modelo mixto generalizado. Además, se realizó un análisis de supervivencia, que reveló que aquellas colmenas con cargas elevadas de DWV y

varroa eran más proclives a colapsar, sobre todo tras un periodo de al menos cuatro meses manteniendo niveles altos de estos dos patógenos.

Por lo tanto, el trabajo descrito en el presente capítulo supuso la coalición de los resultados obtenidos en capítulos previos, permitiendo así la validación de los mismos. Además, se ha comprobado la eficacia de la determinación del vigor como método para estimar la fortaleza de la colmena, en comparación con el método estandarizado.

# Abstract

Many factors, including pathogens, contribute to the continuing losses of colonies of the honey bee *Apis mellifera,* which has led to steady population decline. In particular, colony losses have been linked to deformed wing virus (DWV) and the *Varroa destructor* mite.

To clarify the potential role of these two pathogens in honey bee colony weakening and loss, we sampled colonies in southern Spain during a 21-month period and analyzed the samples for loads of four viruses and varroa. Loads of DWV and black queen cell virus as well as varroa infestation negatively correlated with colony vigor as measured using the subjective colony strength method. General mixed model identified varroa and DWV as the main factors involved in colony weakening.

Our results confirm that varroa and DWV play a key role in triggering colony weakening in southern Spain and provide evidence that experienced beekeepers' and technicians' assessments of colony vigor can accurately estimate colony strength.

# 4.1 Introduction

In recent years, high annual losses of honey bee colonies and reduced population of native and wild bees have been reported (Genersch, 2010; Goulson et al., 2015; Antoine Jacques et al., 2017; Ravoet et al., 2013). These losses have been attributed to colony collapse disorder (vanEngelsdorp, Evans, et al., 2009) and winter losses of 10-15% worldwide, with reductions in overall colony health, brood density and total honeybee comb number in some cases (Zee et al., 2014), or even higher ratio of brood to bees (compensatory brood-production by diseased colonies) (Wegener et al., 2016). Meanwhile colony losses has reached a level of 30.7 % in 2018 in the USA (Bruckner et al., 2018), European colony losses mean mortality has remained lower (11.22% in 2014) (Jacques et al., 2016). These losses appear to result from several factors, including pathogens, nutrition, beekeeping management, environment and pesticides (vanEngelsdorp, Evans, et al., 2009), as well as colony collapse disorder (CCD) and overwintering (Kielmanowicz et al., 2015).

While a combination of intrinsic and environmental stressors perturbs the equilibrium among honey bee, pathogens and environment, hive decline appears to depend strongly on pathogens (vanEngelsdorp, Evans, et al., 2009). Different combinations of pathogens have been identified in dead or depopulated colonies (Dainat et al., 2012a; Goulson et al., 2015; vanEngelsdorp, Evans, et al., 2009), where pathogens seem to interact through synergy (mutual facilitation) or antagonism (inhibition), or the pathogens may not interact but instead cause additive (cumulative) effects (Wegener et al., 2016). Direct effects of pathogen combinations in the same colony are still poorly understood and may depend on host physiology and apiary environment. For example, varroa seasonality influences load of varroa-related viruses, so the effects of pathogens on honey bee health may vary with the seasons (Francis, Nielsen, & Kryger, 2013; Tentcheva et al., 2004).

Widespread all over the world, the *Varroa destructor* mite has adapted to the developmental stages of the honey bee and is a recurrent threat to honey bee populations (Ball, 1993). The mite externally digest and consume fat body tissue (Ramsey et al., 2019), and feeds on honey bee haemolymph, causing weight loss at individual level. It also damages honey bees indirectly by transmitting several viruses, including deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood bee virus (SBV) and Israeli acute paralysis virus (IAPV) (Kralj & Fuchs, 2006; Martin et al., 2012). Meta-genomic analysis has suggested that IAPV may cause CCD (Cox-Foster et al., 2007; Maori et al., 2007), but subsequent work in Spanish apiaries has called into question the link between IAPV and colony losses (Blanchard et al., 2008; Garrido et al., 2013; Vicente-Rubiano, Kukielka, de las Heras, & Sánchez-Vizcaíno, 2013).

*Varroa destructor* mite can synergise with the DWV picornavirus to trigger colony losses (Nazzi et al., 2012). When varroa infestation levels exceed 2,000-3,000 mites per colony during the autumn season, most over-wintering bees become susceptible to DWV infection, causing colony collapse during the winter. This is because the new brood produced during this period is insufficient to replace the infected over-wintering bees that die (Martin, 2001). Even when larvae affected by DWV and varroa survive, they show altered behavior and learning capacity as well as shorter life span (Benaets et al., 2017; Iqbal & Mueller, 2007; Nazzi & Pennacchio, 2018).

Parasites can reduce colony strength and vigour, rendering the colony more vulnerable to stress-induced disease. Colony strength is typically measured objectively based on certain parameters or subjectively based on visual estimation of a target by one or more observers (Delaplane et al., 2013b). These assessments require opening the hive, removing all frames and allowing substantial time for at least two experts to assess the relevant parameters. Given the difficulty of such assessments in field conditions, beekeepers can instead measure colony vigour, which is defined as strength and health of a living organism (the colony). It can be

assessed simply by determination of bee´s activity (visual inspection, emitted sounds) (Hatjina et al., 2014).

Load of DWV and levels of varroa can predict colony collapse, and both agents are linked to colony strength (Dainat et al., 2012b; Sumpter & Martin, 2004a). Although this fact has been already reported by previous studies (Francis et al., 2013; Koziy et al.; Martin, 2001; Nazzi et al., 2012), few studies have demonstrated this relationship between the mite and the virus with colony weakness in Spain. Most of the works have focused on determining their prevalence without directly linked both pathogens to colony death or weakness. Understanding the dynamics of DWV and varroa and their relationship to colony strength may provide information useful for colony monitoring. This was the objective of the present study, which relied on colony vigour as an alternative way to determine colony strength. Ten *Apis mellifera* colonies in an experimental apiary were sampled during a 21-month period, and samples were evaluated for levels of four viruses and varroa mite. Colony strength and vigour were quantified during sampling. We hypothesised that higher viral load and varroa infestation levels would be associated with poor vigour, low population, lack of brood, and reduced honey and pollen reserves.

# 4.2 Material and methods

## 4.2.1 Experimental setup

The colonies sampled in this study were located in Andalusia in southeast Spain, which contains the second largest number of hives in the country (20% of the colonies in the country) and is one of the country's most honey-productive regions (around 6 colonies/Km$^2$). Technicians from the Beekeeping Reference Centre of Andalusia at the University of Córdoba maintained and monthly sampled an experimental *Apis mellifera* apiary from March 2015 to March 2017, except for July and August 2015, when sampling was not possible. In the beginning, the apiary

contained 10 colonies, of which seven collapsed during the study: one colony at May 2015; another colony at June 2015; another colony at August 2015; another colony at April 2016; two colonies at December 2016; and one colony at January 2017. A total of 21 samplings were monthly conducted, generating 142 samples. During sampling, epidemiological data were collected based on colony-level inspection.

### 4.2.2 Determination of colony strength by a standardized method (subjective method)

At each sampling, colony strength was measured by two experienced technicians estimating the percentages of cells in each comb that were covered by bees, brood, honey or pollen, following the subjective method as described (Delaplane, van der Steen, & Guzman-Novoa, 2013a). Raw data were converted into colony bee populations as described (Delaplane et al., 2013b).

### 4.2.3 Determination of colony strength by vigour measurement

At each sampling, two experienced technicians assessed colony vigour on a scale from 1 (least vigorous) to 10 (most vigorous). A score of 1 was associated with small population, low production, and fewer than three inter-panel spaces full of bees. A score of 10 was associated with large population, high production, and more than 6-7 inter-panel spaces between frames full of bees. Worse health status indicators included obvious presence of clear DWV disease symptoms, such as deformed wings and shortened abdomen and discolouration; diagnosis of any disease potentially associated with immune depletion, such as chalkbrood (Glinski, 2003); problems in managing *Varroa destructor* infestation; presence of dead bees around the colony; and a clearly reduction of honey bees.

### 4.2.4 Quantification of bee viruses

Samples of adult bees were taken from the hive entrance of each colony and frozen at -80 °C until analysis. Each sample consisted of a pool of 10 bees, which were homogenised in 5 mL of sterile phosphate-buffered saline (PBS) using a mortar and pestle. This amount of starting material should allow detection of DWV if it is present in more than 25% of bees, with a detection probability of 99% at the colony level (Pirk et al., 2013). RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and stored at −80 °C. This RNA served as template in one-step real-time reverse transcription polymerase chain reaction based on SYBR Green detection for the following viruses: DWV, black queen cell virus (BQCV) (Kukielka, Esperón, et al., 2008), Israeli acute paralysis virus (Maori et al., 2009), and sacbrood bee virus (SBV) (Amiri et al., 2015). Viral loads were classified in four categories according to (Amiri et al., 2015).

Loads were quantified in absolute terms. RT-PCR and PCR amplification products of bee pathogens were cloned into a pGemT® vector (Promega, Madison, WI, USA) and transformed into OneShot® TOP10 chemically competent cells (Invitrogen, Carlsbad, California, USA) according to the manufacturers' instructions. Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Venlo, Netherlands) and confirmed as having pathogen-specific inserts using PCR. Plasmid concentrations (ng/µl) were determined using the Nanodrop® apparatus (Thermo Fisher Scientific Waltham, Massachusetts, USA). Standard curves were constructed using triplicate measurements of serial dilutions of known amounts of each plasmid, ranging from $10^{10}$ to $10^{1}$ copies/bee (limit of quantitation = $10^{1}$ copies/bee). Samples for which the dissociation curves showed the same Tm as positive controls but whose Ct values were below the limit of quantitation were considered positive but not quantifiable (Gauthier et al., 2007; Ribière et al., 2002). A virus-free (negative control)

and a virus-infected (positive control) sample were included on each assay. Pathogen load was expressed as genome equivalent copies per bee (GEC/bee).

### 4.2.5 Quantification of *Varroa destructor* infestation

*Varroa destructor* load was quantified in all colonies at each monthly sampling throughout the study period, except for July and August 2015, when sampling was not possible. Mite load was quantified using the soapy water method (Dietemann, D Ellis, et al., 2013). Briefly, 300 bees were collected from the colony and shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. *Varroa destructor* infestation rate was calculated as follows:

*Varroa destructor* infestation rate = (no. mites /100 bees counted)

After sampling and inspection in September 2015/2016 and March 2016, colonies were treated with oxalic acid against varroa mites.

### 4.2.6 *Nosema ceranae* testing

As *Nosema ceranae* has been related to colony mortality in Spanish apiaries, a subset of the colonies were screened for this pathogen. 57 of the 142 samples, which corresponded to seven of the ten colonies in the period between March 2015 and April 2016, were examined for *Nosema ceranae*, as described in (Barroso-Arévalo et al., 2019). *Nosema apis* was not evaluated because of its low prevalence in Spain (MAPA, 2017).

### 4.2.7 Statistical analyses

All data were codified into six categorical variables (Colony ID, sampling, season, death, DWV level, varroa level) and eight continuous variables (DWV load, BQCV load, *Varroa destructor* infestation rate, bee area, brood area, honey area, pollen

area, colony vigour). Viral loads were $log_{10}$-transformed in order to make patterns more visible.

All statistical analyses were performed using SPSS 22 (IBM, Chicago, USA) and R software for statistical computing, v. 3.1.0 (R Development Core Team 2014). All statistical tests were set at a significance level of 95%; i.e. p-value below 0.05.First, descriptive analysis focused on an exploratory study of the potential relationships among pathogen loads and between pathogen loads and bee/brood/honey/pollen areas though the Spearman's correlation coefficient (ρ).

Second analysis consisted of survival analysis for investigating the effect of different DWV loads and varroa infestation rates upon the time of the colonies' collapse. For this analysis, DWV load and varroa infestation rate were classified as categorical variables: high DWV load (RNA equivalents/bee$\geq 10^6$), low DWV load ($10^6$<RNA equivalents/bee); high varroa infestation rate (varroa infestation rate >3%) and low varroa infestation rate (varroa infestation rate ≤3%) (Bulacio, 2011). As DWV load and varroa infestation rate varied over time, colonies were sub classified into periods according to their health status based on the classification explained above. For instance, if colony 1 showed high DWV load and low varroa infestation rate for three months, it was considered as one independent period (1a); if colony 1 showed low DWV load and low varroa infestation rate in the following four months, it was considered as another period (1b). Thus, each colony was transformed into different analysis units. This approach allowed us to study the probability of colony collapse in response to variations in DWV and varroa levels. Two Kaplan-Meier curves were created to evaluate the cumulative risk of colony collapse with different DWV load (high/low) and varroa infestation rate (high/low). In addition, Cox regression analysis was performed to detect significant differences in the risk of colony collapse depending on DWV and varroa levels. Colony was considered as a strata variable for the analysis. Cox regression analysis was computed with the survival and survminer packages in R software.

Third analysis focused on exploring the potential influence of DWV load, BQCV load, or varroa infestation rate on colony vigour. A generalized linear mixed model (GLMM) with colony as random effect was conducted between the outcome DWV and BQCV load (quantitative variable: RNA equivalents/bee) and colony vigour. The same analysis was performed with the outcome varroa infestation level (quantitative variable: no mites/100 bees counted) and vigour. The random effects examine the difference between colonies with respect to their baseline pathogen level and the changes over time. For this analysis, vigour was classified as a binary variable. As the values for vigour were ranged between 3 and 9, vigour was classified as low if the vigour value was < 6 (Vigour = 1), or high if the vigour value was ≥ 6 (Vigour = 0). GLMM was computed with the lm4 package in R software.

# 4.3 Results

## 4.3.1 Pathogen loads and *Varroa destructor* infestation rate during the study

All samples evaluated for *Nosema ceranae* tested negative. The results showed a high prevalence of DWV and BQCV, while SBV and IAPV were not detected. DWV was present in 139 of 142 samples (97.8%), with an average of 3.39 x $10^5$ GEC/bee (range, 8.32 GEC/bee to 5.25 x $10^9$ GEC/bee). BQCV was present in 113 of 142 samples (79.8%), with an average of 1.58 x $10^3$ GEC/bee (range, 22.1 GEC/µL to 9.83 x $10^9$ GEC/bee). Varroa was detected in at least 80% of samples from all colonies. It was present in 119 of 142 samples (83.8%), achieving its higher levels in September and October. Treatments successfully decreased varroa levels (Figure 1). Comparison between varroa levels and number of brood combs showed that varroa also decreased its levels when the brood dropped (Figure 1).

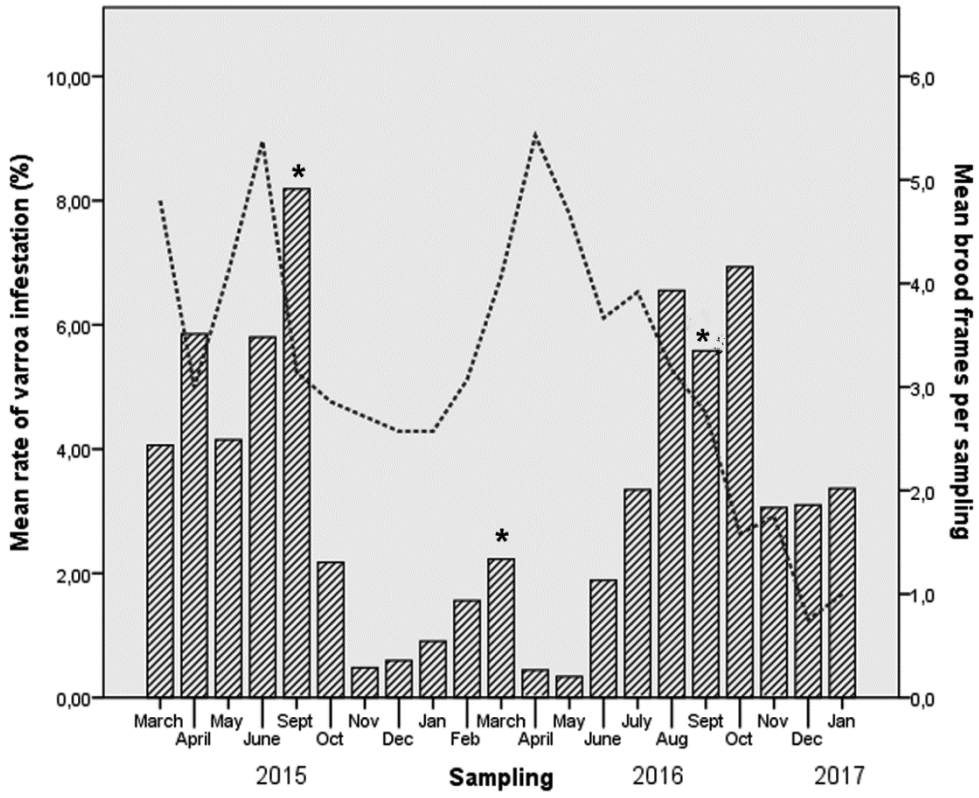


**Figure 1.** Rates of *Varroa destructor* infestation (per 100 adult bees) and mean of number of brood frames evolution in each colony, based on monthly sampling. Asterisks mark months when anti-*Varroa destructor* treatment was applied.

### 4.3.2 Correlations among *Varroa destructor* infestation rate, pathogen loads and colony strength

Significant correlations are shown in Table 1. Correlation between *Varroa destructor* infestation rate and DWV load is shown in Figure 2.

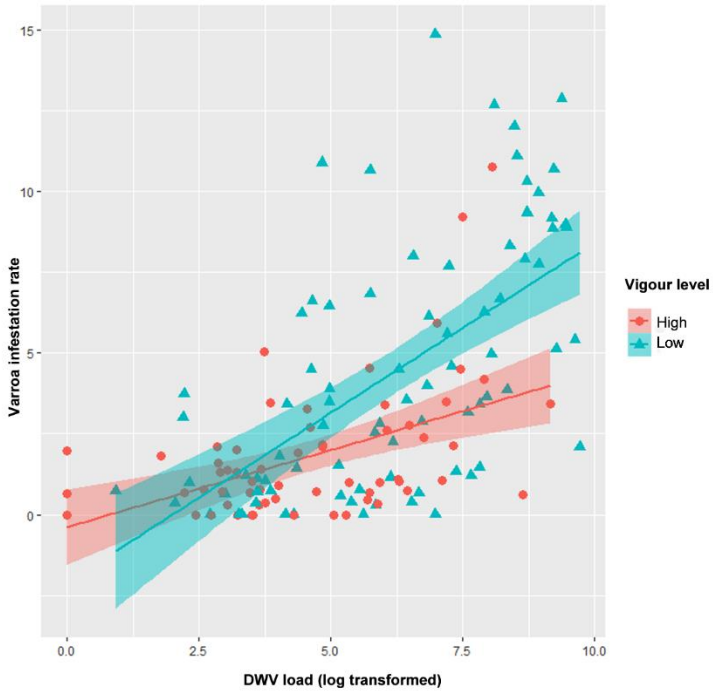


**Figure 2.** Scatter plot showing correlation of DWV load and varroa infestation rate. Low-vigor colonies are shown in blue; high-vigor colonies, red. Regression lines and confident bands are also shown.

### 4.3.3 Survival analysis

Kaplan-Meier plots are showed in Figure 3. Cox regression model showed that DWV load (p-value = 0.0166) and varroa infestation rate (p-value = 0.0481) were independent influencing factors affecting colony collapse. High DWV load was associated with a significantly increased mortality risk (Hazzard Risk, 13.48; 95% confident interval), as well as high varroa infestation rate (HR, 63.95; 95% CI). Most of the colonies collapsed after four months with high DWV load and high varroa infestation rate.

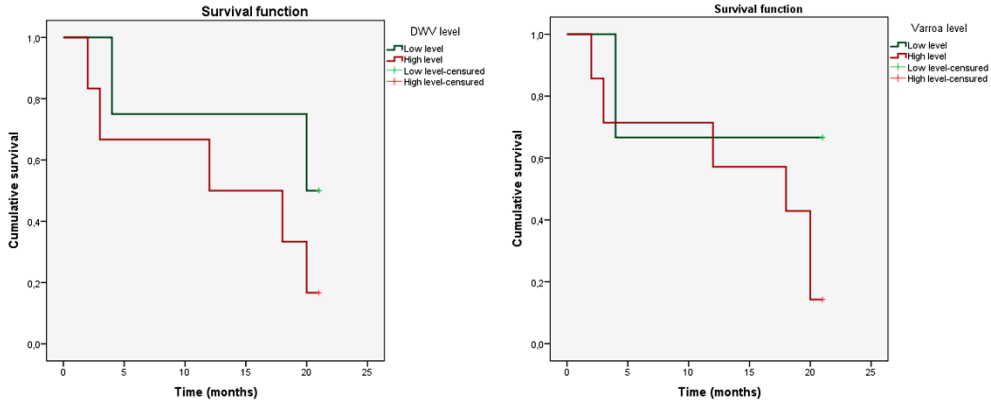


**Figure 3.** Overall survival according to different DWV **(A)** and *Varroa destructor* **(B)** levels. Green color represents low levels of pathogen; red color represents high levels of pathogens.

### 4.3.3 Generalized mixed model to examine potential relationships of virus loads or varroa infestation rate with colony vigor

Colony vigor was significantly associated with DWV load and Varroa infestation rate, but not with BQCV load. Varroa infestation rate was significantly higher in those colonies with reduced vigor ($t = 1.303$, $p = 0.007$) as well as DWV load ($t = 1.167$, $p = 0.017$).

### 4.3.4 Presence of disease symptoms

Some colonies showed symptoms of DWV (such as deformed wings and shortened abdomen and discolouration) and ascospherosis and/or excessive presence of varroa in the phoretic stage, especially in December, January and February. However, the presence of DWV symptoms or ascospherosis did not correlate significantly with viral load or varroa infestation rate.

# 4.4 Discussion

In the present study we analysed relationships among the loads of four viruses and *Varroa destructor* mite, colony vigour, colony strength (in terms of bee/brood/honey/pollen area), and presence of symptoms in an experimental apiary during a 21-month period from March 2015 to January 2017.

RT-qPCR analysis revealed a high prevalence of DWV and BQCV, while IAPV and SBV were not detected. The total absence of IAPV during the entire study suggests that the virus is not abundant in Spain, at least not in the province of Córdoba. The fact that we can exclude IAPV as a cause of the total loss and depopulation of several colonies during the study is reminiscent of our 2013 observation, also in Andalusia, that IAPV was not involved with colony collapse in that study (Marina Vicente-Rubiano et al., 2013). Our results suggest that the association between IAPV and CCD observed in the USA (Cox-Foster et al., 2007; Maori et al., 2007) may not apply to southern Spain.

BQCV was quite prevalent in our study. This result agrees with those obtained previously in Andalusia and other parts of Spain (Cepero et al., 2014; Kukielka, Perez, et al., 2008). DWV was found in the majority of samples, which is higher than the prevalence previously reported in northern and central Spain (Gisder et al., 2009), but agrees with the results from the annual report about honey bee losses performed in the country, where DWV was found in a 99% of the apiaries analysed (MAPA, 2017). The low prevalence in that previous work may be related to the low presence of *V. destructor* in those apiaries. In our study, varroa was found in 83.8% of samples. This apparent correlation between DWV load and varroa infestation rate agrees with previous studies in Spain (Kukielka, Esperón, et al., 2008; Vicente-Rubiano, 2015). Varroa have to injure honey bees directly by consuming body tissue (Ramsey et al., 2019) and feeding on their haemolymph, and it can impact bees by transmitting viruses and activating viral replication (Kralj & Fuchs, 2006; Yang & Cox-Foster,

2005). The combination of high DWV load in bees during the summer-autumn season may reflect varroa dynamics and reproduction inside brood cells. As the number of brood increases from spring to summer, mite reproduction increases, peaking in late summer (Evans & Cook, 2018). These considerations suggest that DWV and varroa may act together to affect colony health (Di Prisco et al., 2016), and that these negative effects are likely to be greater when brood is growing, since varroa is reproducing and thereby helping DWV to replicate (Wegener et al., 2016). Virus-parasite-host immunity synergism can profoundly alter colony response to pathogens (Di Prisco et al., 2016; Nazzi et al., 2012). Cox regression analysis and Kaplan-Meier curves showed that after short periods (between two or four months) with high DWV load and high varroa infestation rate, colonies were more likely to collapse. This fact emphasise the need to monitor DWV and varroa levels in apiaries, as a preventive measure.

We found that colonies that died during the study period showed significantly lower bee and brood areas and significantly lower vigour than colonies that survived. Vigour negatively correlated with varroa infestation rate and DWV load, as well as DWV and brood area, suggesting that the mite-virus complex may predispose the colony to depopulation and weakness. However, these results need to be interpreted with caution, since correlation coefficients were low for those variables (Table 1) and, therefore, their value may be limited. Generally, colonies with high varroa infestation and DWV infection are more likely to experience stress and are therefore more vulnerable to depopulation and less vigour. BQCV also negatively correlated with colony vigour. Since BQCV infection is often asymptomatic and therefore covert (Tentcheva et al., 2004), this correlation may reflect immunosuppression caused by other factors, such as varroa infestation or DWV infection. This immunosuppression can facilitate BQCV replication. In this way, opportunistic viruses like BQCV may actively replicate in bee brood that is infected with DWV and infested with varroa, leading to strong BQCV infection in adult bees (Chen & Siede, 2007; Gauthier et al., 2007).

We ran a generalized linear mixed model to determine whether viral load and varroa infestation rate could explain the observed decreases in colony vigour. The model suggested that, indeed, high varroa infestation rate and high DWV load were related to reduced colony vigour and therefore unstable health status. These results confirm several previous studies documenting a relationship between DWV and the mite (Bowen-Walker et al., 1999; Dainat et al., 2012a; Locke et al., 2014; Martin, 2001). This interaction appears to involve a synergy mediated by host immunity (Nazzi et al., 2012), which is consistent with our observation of overt symptomatology of varroosis and wing deformities in the joint presence of DWV and varroa. Therefore, exhaustive measures to control varroa should be implemented in order to decrease DWV infection.

Seasonal trends were detected for varroa infestation: the mite reached its major infestation level in the spring-summer season, suggesting that the mite contributes to pathogen presence and replication during this season (Yang & Cox-Foster, 2005). Bee/brood/pollen/honey areas were also higher in this season, which may help the mite expand rapidly to the colonies (Nurnberger, Hartel, & Steffan-Dewenter, 2019; Wegener et al., 2016).

Pollen area negatively correlated with varroa infestation rate. A decline in pollen harvest has been associated with a direct reduction in brood production, which may have a negative impact on the adult bee population size, and lack of honey reserves before overwintering. Consequently, colony health can be affected, making the colony more vulnerable to diseases like varroosis (Requier, Odoux, Henry, & Bretagnolle, 2017). Individual bees infested with varroa during their development usually survive to emergence but may show signs of physical or physiological damage as adults (Barron, 2015). The feeding and reproduction of varroa mites on developing larvae and pupae of worker brood may have a major effect on adult bee activity (Dainat et al., 2012b; Requier et al., 2017). The mite can trigger wing deformities as well as behavioral alterations that reduce foraging activity, reducing pollen

collection. A recent study have suggested that pollen supplementation may provide the survival of honey bee colonies infested by the parasite (Annoscia et al., 2017). Therefore, practical use of pollen for the prevention of varroa infestation should be further explored.

We found that assessment of colony strength measured subjectively (Delaplane et al., 2013b) gave similar results as assessment of colony vigour. In addition, vigour correlated positively with bees/brood/pollen/honey area, suggesting that vigour estimation by an experienced technician may be a more straightforward and less invasive method for estimating colony strength.

# 4.5 Conclusions

We showed that DWV, BQCV and varroa were highly prevalent at all samplings. DWV load correlated with varroa infestation rate, and high load of both agents was related to lower colony vigour. While our results suggest that the mite-virus interaction contributes to colony weakening, more studies are needed to examine whether interaction with other pathogens also plays a role.

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# High Load of Deformed Wing Virus and *Varroa destructor* Infestation Are Related to Weakness of Honey Bee Colonies in Southern Spain

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Many factors, including pathogens, contribute to the continuing losses of colonies of the honey bee *Apis mellifera*, which has led to steady population decline. In particular, colony losses have been linked to deformed wing virus (DWV) and the *Varroa destructor* mite. To clarify the potential role of these two pathogens in honey bee colony weakening and loss, we sampled colonies in southern Spain during a 21-month period and analyzed the samples for loads of four viruses and varroa. Loads of DWV and black queen cell virus as well as varroa infestation negatively correlated with colony vigor as measured using the subjective colony strength method. Logistic regression identified varroa and DWV as the main factors involved in colony weakening. Our results confirm that varroa and DWV play a key role in triggering colony weakening in southern Spain and provide evidence that experienced beekeepers' and technicians' assessments of colony vigor can accurately estimate colony strength.

**Keywords: deformed wing virus, *Varroa destructor* mite, colony strength, honey bee, colony vigor, colony losses**

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## INTRODUCTION

In recent years, high annual losses of honey bee colonies and reduced population of native and wild bees have been reported (Genersch, 2010; Ravoet et al., 2013; Goulson et al., 2015; Jacques et al., 2017). These losses have been attributed to colony collapse disorder (vanEngelsdorp et al., 2009) and winter losses of 10–15% worldwide, with reductions in overall colony health, brood density, and total honeybee comb number in some cases (Zee et al., 2014), or even higher ratio of brood to bees (compensatory brood production by diseased colonies; Wegener et al., 2016). Meanwhile, colony losses have reached a level of 30.7% in 2018 in the USA (Bruckner et al., 2018), and mean mortality of European colony losses has remained lower (11.22% in 2014) (Jacques et al., 2016). These losses appear to result from several factors, including pathogens, nutrition, beekeeping management, environment, and pesticides (vanEngelsdorp et al., 2009), as well as colony collapse disorder (CCD) and overwintering (Kielmanowicz et al., 2015).

While a combination of intrinsic and environmental stressors perturbs the equilibrium among honey bee, pathogens, and environment, hive decline appears to depend strongly on pathogens

(vanEngelsdorp et al., 2009). Different combinations of pathogens have been identified in dead or depopulated colonies (vanEngelsdorp et al., 2009; Dainat et al., 2012a; Goulson et al., 2015), where pathogens seem to interact through synergy (mutual facilitation) or antagonism (inhibition), or the pathogens may not interact but instead cause additive (cumulative) effects (Wegener et al., 2016). Direct effects of pathogen combinations in the same colony are still poorly understood and may depend on host physiology and apiary environment. For example, varroa seasonality influences load of varroa-related viruses, so the effects of pathogens on honey bee health may vary with the seasons (Tentcheva et al., 2004; Francis et al., 2013).

Widespread all over the world, the *Varroa destructor* mite has adapted to the developmental stages of the honey bee and is a recurrent threat to honey bee populations (Ball, 1993). The mite externally digests and consumes fat body tissue (Ramsey et al., 2019) and feeds on honey bee hemolymph, causing weight loss at individual level. It also damages honey bees indirectly by transmitting several viruses, including deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood bee virus (SBV), and Israeli acute paralysis virus (IAPV) (Kralj and Fuchs, 2006; Martin et al., 2012). Meta-genomic analysis has suggested that IAPV may cause CCD (Cox-Foster et al., 2007; Maori et al., 2007), but subsequent work in Spanish apiaries has called into question the link between IAPV and colony losses (Blanchard et al., 2008; Garrido et al., 2013; Vicente-Rubiano et al., 2013).

*Varroa destructor* mite can synergize with the DWV picornavirus to trigger colony losses (Nazzi et al., 2012). When varroa infestation levels exceed 2,000–3,000 mites per colony during the autumn season, most over-wintering bees become susceptible to DWV infection, causing colony collapse during the winter. This is because the new brood produced during this period is insufficient to replace the infected over-wintering bees that die (Martin, 2001). Even when larvae affected by DWV and varroa survive, they show altered behavior and learning capacity as well as shorter life span (Iqbal and Mueller, 2007; Benaets et al., 2017; Nazzi and Pennacchio, 2018).

Parasites can reduce colony strength and vigor, rendering the colony more vulnerable to stress-induced disease. Colony strength is typically measured objectively based on certain parameters or subjectively based on visual estimation of a target by one or more observers (Delaplane et al., 2013). These assessments require opening the hive, removing all frames, and allowing substantial time for at least two experts to assess the relevant parameters. Given the difficulty of such assessments in field conditions, beekeepers can instead measure colony vigor, which is defined as strength and health of a living organism (the colony). It can be assessed simply by determination of bee's activity (visual inspection, emitted sounds; Hatjina et al., 2014).

Load of DWV and levels of varroa can predict colony collapse, and both agents are linked to colony strength (Sumpter and Martin, 2004; Dainat et al., 2012b). Although this fact has been already reported by previous studies (Martin, 2001; Nazzi et al., 2012; Francis et al., 2013; Koziy et al., 2019), few studies have demonstrated this relationship between the mite and the virus with colony weakness in Spain. Most of the works have focused on determining their prevalence without directly linked both pathogens to colony death or weakness. Understanding the dynamics of DWV and varroa and their relationship to colony strength may provide information useful for colony monitoring. This was the objective of the present study, which relied on colony vigor as an alternative way to determine colony strength. Ten *Apis mellifera* colonies in an experimental apiary were sampled during a 21-month period, and samples were evaluated for levels of four viruses and varroa mite. Colony strength and vigor were quantified during sampling. We hypothesized that higher viral load and varroa infestation levels would be associated with poor vigor, low population, lack of brood, and reduced honey and pollen reserves.

# MATERIALS AND METHODS

## Experimental Setup

The colonies sampled in this study were located in Andalusia in southeast Spain, which contains the second largest number of hives in the country (20% of the colonies in the country) and is one of the country's most honey-productive regions (around 6 colonies/km$^2$). Technicians from the Beekeeping Reference Centre of Andalusia at the University of Córdoba maintained and monthly sampled an experimental *Apis mellifera* apiary from March 2015 to March 2017, except for July and August 2015, when sampling was not possible. In the beginning, the apiary contained 10 colonies, of which 7 collapsed during the study: one colony at May 2015; another colony at June 2015; another colony at August 2015; another colony at April 2016; two colonies at December 2016; and one colony at January 2017. A total of 21 samplings were monthly conducted, generating 142 samples. During sampling, epidemiological data were collected based on colony-level inspection.

## Determination of Colony Strength by a Standardized Method (Subjective Method)

At each sampling, colony strength was measured by two experienced technicians estimating the percentages of cells in each comb that were covered by bees, brood, honey, or pollen, following the subjective method as described (Delaplane et al., 2013). Raw data were converted into colony bee populations as described (Delaplane et al., 2013).

## Determination of Colony Strength by Vigor Measurement

At each sampling, two experienced technicians assessed colony vigor on a scale from 1 (least vigorous) to 10 (most vigorous). A score of 1 was associated with colonies with a small population occupying less than the space between three frames and having low amount of stores, in the form of nectar/honey and pollen. A score of 10 was given to colonies with a large population of bees, occupying more than the space between 6 and 7 frames and having ample stores. Worse health status indicators included obvious presence of clear DWV disease symptoms, such as deformed wings and shortened abdomen and

discoloration; diagnosis of any disease potentially associated with immune depletion, such as chalkbrood (Glinski and Buczek, 2003); problems in managing *Varroa destructor* infestation; presence of dead bees around the colony; and a clearly reduction of honey bees.

## Quantification of Bee Viruses

Samples of adult bees were taken from the hive entrance of each colony and frozen at −80°C until analysis. Each sample consisted of a pool of 10 bees, which were homogenized in 5 ml of sterile phosphate-buffered saline (PBS) using a mortar and pestle. This amount of starting material should allow detection of DWV if it is present in more than 25% of bees, with a detection probability of 99% at the colony level (Delaplane et al., 2013). RNA was extracted using the column-based Nucleospin II Virus® kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and stored at −80°C. This RNA served as template in one-step real-time reverse transcription polymerase chain reaction based on SYBR Green detection for the following viruses: DWV, black queen cell virus (BQCV; Kukielka et al., 2008a), Israeli acute paralysis virus (Maori et al., 2009), and sacbrood bee virus (SBV; Amiri et al., 2015). Viral loads were classified into four categories according to Amiri et al. (2015). DWV primers were previously tested for the detection of a wide variety of DWV.

Loads were quantified in absolute terms. RT-PCR and PCR amplification products of bee pathogens were cloned into a pGemT® vector (Promega, Madison, WI, USA) and transformed into OneShot® TOP10 chemically competent cells (Invitrogen, Carlsbad, California, USA) according to the manufacturers' instructions. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands) and confirmed as having pathogen-specific inserts using PCR. Plasmid concentrations (ng/μl) were determined using the Nanodrop® apparatus (Thermo Fisher Scientific Waltham, Massachusetts, USA). Standard curves were constructed using triplicate measurements of serial dilutions of known amounts of each plasmid, ranging from $10^{10}$ to $10^{1}$ copies/bee (limit of quantitation = $10^{1}$ copies/bee). Samples for which the dissociation curves showed the same Tm as positive controls but whose Ct values were below the limit of quantitation were considered positive but not quantifiable (Ribière et al., 2002; Gauthier et al., 2007). A virus-free (negative control) and a virus-infected (positive control) sample were included on each assay. Pathogen load was expressed as genome equivalent copies per bee (GEC/bee).

## Quantification of *Varroa destructor* Infestation

*Varroa destructor* load was quantified in all colonies at each monthly sampling throughout the study period, except for July and August 2015, when sampling was not possible. Mite load was quantified using the soapy water method (Dietemann et al., 2013). Briefly, 300 bees were collected from the colony and shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. *Varroa destructor* infestation rate was calculated as follows:

$$\textit{Varroa destructor}\ \text{infestation rate} = \left(\text{no. of mites} / 100\ \text{bees counted}\right).$$

After sampling and inspection in September 2015/2016 and March 2016, colonies were treated with oxalic acid against varroa mites.

## *Nosema ceranae* Testing

As *Nosema ceranae* has been related to colony mortality in Spanish apiaries, a subset of the colonies was screened for this pathogen. About 57 of the 142 samples, which corresponded to 7 of the 10 colonies in the period between March 2015 and April 2016, were examined for *Nosema ceranae*, as described in Barroso-Arévalo et al. (2019). *Nosema apis* was not evaluated because of its low prevalence in Spain (Ministerio de agricultura y pesca, a.y.m.a, 2017).

## Statistical Analyses

All data were codified into six categorical variables (Colony ID, sampling, season, death, DWV level, and varroa level) and eight continuous variables (DWV load, BQCV load, *Varroa destructor* infestation rate, bee area, brood area, honey area, pollen area, and colony vigor). Viral loads were $\log_{10}$-transformed in order to make patterns more visible.

All statistical analyses were performed using SPSS 22 (IBM, Chicago, USA) and R software for statistical computing, v. 3.1.0 (R Development Core Team, 2014). All statistical tests were set at a significance level of 95%; i.e., $p < 0.05$. First, descriptive analyses was performed. Potential relationships among pathogen loads and between pathogen loads and bee/brood/honey/pollen areas were explored though the Spearman's correlation coefficient ($\rho$).

Second analysis consisted of survival analysis for investigating the effect of different DWV loads and varroa infestation rates upon the time of the colonies' collapse. For this analysis, DWV load and varroa infestation rate were classified as categorical variables: high DWV load (RNA equivalents/bee $\geq 10^{6}$) and low DWV load ($10^{6}$ > RNA equivalents/bee); high varroa infestation rate (varroa infestation rate > 3%) and low varroa infestation rate (varroa infestation rate ≤ 3%) (Bulacio, 2011). As DWV load and varroa infestation rate varied over time, colonies were subclassified into periods according to their health status based on the classification explained above. For instance, if colony 1 showed high DWV load and low varroa infestation rate for 3 months, it was considered as one independent period (1a); if colony 1 showed low DWV load and low varroa infestation rate in the following 4 months, it was considered as another period (1b). Thus, each colony was transformed into different analysis units. This approach allowed us to study the probability of colony collapse in response to variations in DWV and varroa levels. Two Kaplan-Meier curves were created to evaluate the cumulative risk of colony collapse with different DWV load (high/low)

and varroa infestation rate (high/low). In addition, Cox regression analysis was performed to detect significant differences in the risk of colony collapse depending on DWV and varroa levels. Colony was considered as a strata variable for the analysis. Cox regression analysis was computed with the survival and survminer packages in R software.

Third analysis focused on exploring the potential influence of DWV load, BQCV load, or varroa infestation rate on colony vigor. A generalized linear mixed model (GLMM) with colony as random effect was conducted between the outcome DWV and BQCV load (quantitative variable: RNA equivalents/bee) and colony vigor. The same analysis was performed with the outcome varroa infestation level (quantitative variable: no. of mites/100 bees counted) and vigor. The random effects examine the difference between colonies with respect to their baseline pathogen level and the changes over time. For this analysis, vigor was classified as a binary variable. As the values for vigor were ranged between 3 and 9, vigor was classified as low if the vigor value was <6 (Vigor = 1) or high if the vigor value was ≥6 (Vigor = 0). GLMM was computed with the lm4 package in R software.

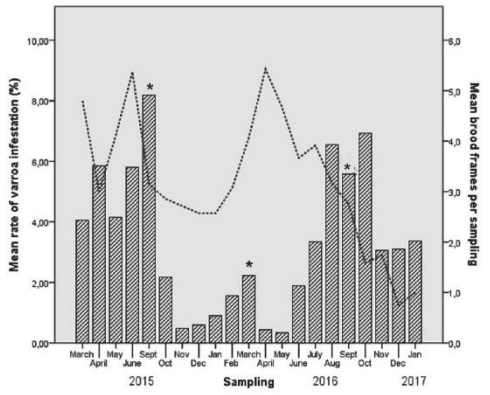


**FIGURE 1** | Rates of *Varroa destructor* infestation (per 100 adult bees) and mean of number of brood frames evolution in each colony, based on monthly sampling. Asterisks mark months when anti-*Varroa destructor* treatment was applied.

# RESULTS

## Pathogen Loads and *Varroa destructor* Infestation Rate During the Study

All samples evaluated for *Nosema ceranae* tested negative. The results showed a high prevalence of DWV and BQCV, while SBV and IAPV were not detected. DWV was present in 139 of 142 samples (97.8%), with an average of $3.39 \times 10^5$ GEC/bee (range, 8.32 GEC/bee to $5.25 \times 10^9$ GEC/bee). BQCV was present in 113 of 142 samples (79.8%), with an average of $1.58 \times 10^3$ GEC/bee (range, 22.1 GEC/µl to $9.83 \times 10^9$ GEC/bee). Varroa was detected in at least 80% of samples from all colonies. It was present in 119 of 142 samples (83.8%), achieving its higher levels in September and October. Treatments successfully decreased varroa levels (**Figure 1**). Comparison between varroa levels and number of brood combs showed that varroa also decreased its levels when the brood dropped (**Figure 1**).

**TABLE 1** | Significant correlations among variables in the study.

| Correlated variables | *p* | Spearman's correlation coefficient ($\rho$) |
|---|---|---|
| BQCV, brood area | 0.009 | −0.305 |
| BQCV, vigor | 0.031 | −0.252 |
| DWV, brood area | 0.003 | −0.378 |
| DWV, vigor | <0.001 | −0.464 |
| DWV, varroa | <0.001 | 0.616 |
| Varroa, pollen area | 0.011 | −0.306 |
| Varroa, vigor | <0.001 | −0.474 |
| Bee area, brood area | <0.001 | 0.610 |
| Bee area, honey area | <0.001 | 0.686 |
| Bee area, pollen area | <0.001 | 0.420 |
| Bee area, vigor | <0.001 | 0.810 |
| Brood area, honey area | 0.038 | 0.210 |
| Brood area, pollen area | 0.002 | 0.317 |
| Brood area, vigor | <0.001 | 0.747 |
| Honey area, pollen area | <0.001 | 0.506 |
| Honey area, vigor | <0.001 | 0.494 |
| Pollen area, vigor | <0.001 | 0.390 |

## Correlations Among *Varroa destructor* Infestation Rate, Pathogen Loads, and Colony Strength

Significant correlations are shown in **Table 1**. Correlation between *Varroa destructor* infestation rate and DWV load is shown in **Figure 2**.

## Survival Analysis

Kaplan-Meier plots are showed in **Figure 3**. Cox regression model showed that DWV load ($p = 0.0166$) and varroa infestation rate ($p = 0.0481$) were independent influencing factors affecting colony collapse. High DWV load was associated with a significantly increased mortality risk (hazard risk, 13.48; 95% confident interval) as well as high varroa infestation rate (HR, 63.95; 95% CI). Most of the colonies collapsed after 4 months with high DWV load and high varroa infestation rate.

## Generalized Mixed Model to Examine Potential Relationships of Virus Loads or Varroa Infestation Rate With Colony Vigor

Colony vigor was significantly associated with DWV load and *Varroa* infestation rate, but not with BQCV load. Varroa infestation rate was significantly higher in those colonies with reduced vigor ($t = 1.303$, $p = 0.007$) as well as DWV load ($t = 1.167$, $p = 0.017$).

## Presence of Disease Symptoms

Some colonies showed symptoms of DWV (such as deformed wings and shortened abdomen and discoloration) and ascospherosis and/or excessive presence of varroa in the phoretic stage, especially in December, January, and February. However, the presence of DWV symptoms or ascospherosis did not correlate significantly with viral load or varroa infestation rate.

# DISCUSSION

In the present study, we analyzed relationships among the loads of four viruses and *Varroa destructor* mite, colony vigor, colony strength (in terms of bee/brood/honey/pollen area), and presence of symptoms in an experimental apiary during a 21-month period from March 2015 to January 2017.

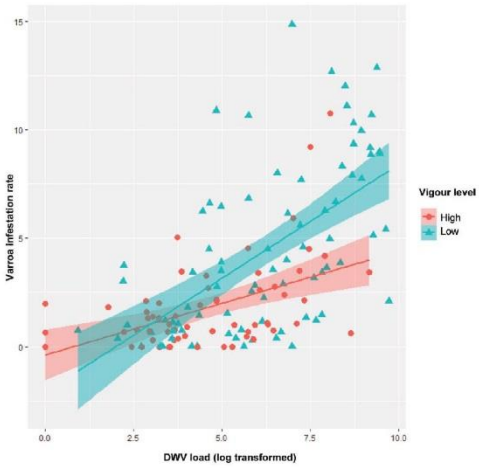


**FIGURE 2 |** Scatter plot showing correlation of DWV load and varroa infestation rate. Low-vigor colonies are shown in blue; high-vigor colonies, red. Regression lines and confident bands are also shown.

RT-qPCR analysis revealed a high prevalence of DWV and BQCV, while IAPV and SBV were not detected. The total absence of IAPV during the entire study suggests that the virus is not abundant in Spain, at least not in the province of Córdoba. The fact that we can exclude IAPV as a cause of the total loss and depopulation of several colonies during the study is reminiscent of our 2013 observation, also in Andalusia, that IAPV was not involved with colony collapse in that study (Vicente-Rubiano et al., 2013). Our results suggest that the association between IAPV and CCD observed in the USA (Cox-Foster et al., 2007; Maori et al., 2007) may not apply to southern Spain.

BQCV was quite prevalent in our study. This result agrees with those obtained previously in Andalusia and other parts of Spain (Kukielka et al., 2008b; Cepero et al., 2014). DWV was found in the majority of samples, which is higher than the prevalence previously reported in northern and central Spain (Gisder et al., 2009), but agrees with the results from the annual report about honey bee losses performed in the country, where DWV was found in a 99% of the apiaries analyzed (Ministerio de agricultura y pesca, a.y.m.a, 2017). The low prevalence in that previous work may be related to the low presence of *V. destructor* in those apiaries. In our study, varroa was found in 83.8% of samples. This apparent correlation between DWV load and varroa infestation rate agrees with previous studies in Spain (Kukielka et al., 2008a; Vicente-Rubiano, 2015). Varroa have to injure honey bees directly by consuming body tissue (Ramsey et al., 2019) and feeding on their hemolymph, and it can impact bees by transmitting viruses and activating viral replication (Yang and Cox-Foster, 2005; Kralj and Fuchs, 2006). The combination of high DWV load in bees during the summer-autumn season may reflect varroa dynamics and reproduction inside brood cells. As the number of brood increases from spring to summer, mite reproduction increases, peaking in late summer (Evans and Cook, 2018). These considerations suggest that DWV and varroa may act together to affect colony health (Di Prisco et al., 2016) and that these negative effects are likely

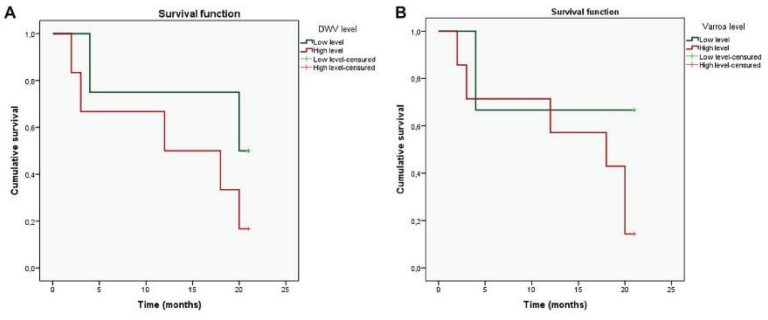


**FIGURE 3 |** Overall survival according to different DWV **(A)** and *Varroa destructor* **(B)** levels. Green color represents low levels of pathogen; red color represents high levels of pathogens.

to be greater when brood is growing, since varroa is reproducing and thereby helping DWV to replicate (Wegener et al., 2016). Virus-parasite-host immunity synergism can profoundly alter colony response to pathogens (Nazzi et al., 2012; Di Prisco et al., 2016). Cox regression analysis and Kaplan-Meier curves showed that after short periods (between 2 and 4 months) with high DWV load and high varroa infestation rate, colonies were more likely to collapse. This fact emphasize the need to monitor DWV and varroa levels in apiaries, as a preventive measure.

We found that colonies that died during the study period showed significantly lower bee and brood areas and significantly lower vigor than colonies that survived. Vigor negatively correlated with varroa infestation rate and DWV load, as well as DWV and brood area, suggesting that the mite-virus complex may predispose the colony to depopulation and weakness. However, these results need to be interpreted with caution, since correlation coefficients were low for those variables (**Table 1**) and, therefore, their value may be limited. Generally, colonies with high varroa infestation and DWV infection are more likely to experience stress and are therefore more vulnerable to depopulation and less vigor. BQCV also negatively correlated with colony vigor. Since BQCV infection is often asymptomatic and therefore covert (Tentcheva et al., 2004), this correlation may reflect immunosuppression caused by other factors, such as varroa infestation or DWV infection. This immunosuppression can facilitate BQCV replication. In this way, opportunistic viruses like BQCV may actively replicate in bee brood that is infected with DWV and infested with varroa, leading to strong BQCV infection in adult bees (Chen and Siede, 2007; Gauthier et al., 2007).

We ran a generalized linear mixed model to determine whether viral load and varroa infestation rate could explain the observed decreases in colony vigor. The model suggested that, indeed, high varroa infestation rate and high DWV load were related to reduced colony vigor and therefore unstable health status. These results confirm several previous studies documenting a relationship between DWV and the mite (Bowen-Walker et al., 1999; Martin, 2001; Dainat et al., 2012a; Locke et al., 2014). This interaction appears to involve a synergy mediated by host immunity (Nazzi et al., 2012), which is consistent with our observation of overt symptomatology of varroosis and wing deformities in the joint presence of DWV and varroa. Therefore, exhaustive measures to control varroa should be implemented in order to decrease DWV infection.

Seasonal trends were detected for varroa infestation: the mite reached its major infestation level in the spring-summer season, suggesting that the mite contributes to pathogen presence and replication during this season (Yang and Cox-Foster, 2005). Bee/brood/pollen/honey areas were also higher in this season, which may help the mite expand rapidly to the colonies (Wegener et al., 2016; Nurnberger et al., 2019).

Pollen area negatively correlated with varroa infestation rate. A decline in pollen harvest has been associated with a direct reduction in brood production, which may have a negative impact on the adult bee population size, and lack of honey reserves before overwintering. Consequently, colony health can be affected, making the colony more vulnerable to diseases like varroosis (Requier et al., 2017). Individual bees infested with varroa during their development usually survive to emergence but may show signs of physical or physiological damage as adults (Barron, 2015). The feeding and reproduction of varroa mites on developing larvae and pupae of worker brood may have a major effect on adult bee activity (Dainat et al., 2012b; Requier et al., 2017). The mite can trigger wing deformities as well as behavioral alterations that reduce foraging activity, reducing pollen collection. A recent study has suggested that pollen supplementation may provide the survival of honey bee colonies infested by the parasite (Annoscia et al., 2017). Therefore, practical use of pollen for the prevention of varroa infestation should be further explored.

We found that assessment of colony strength measured subjectively (Delaplane et al., 2013) gave similar results as assessment of colony vigor. In addition, vigor correlated positively with bees/brood/pollen/honey area, suggesting that vigor estimation by an experienced technician may be a more straightforward and less invasive method for estimating colony strength.

## CONCLUSIONS

We showed that DWV, BQCV, and varroa were highly prevalent at all samplings. DWV load correlated with varroa infestation rate, and high load of both agents was related to lower colony vigor. While our results suggest that the mite-virus interaction contributes to colony weakening, more studies are needed to examine whether interaction with other pathogens also plays a role.

## DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## AUTHOR CONTRIBUTIONS

SB-A, JG, and JS-V conceived this research and designed the experiments. FP and EC participated in the design and interpretation of the data. SB-A and FR performed the experiments and analysis. SB-A and EC wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript.

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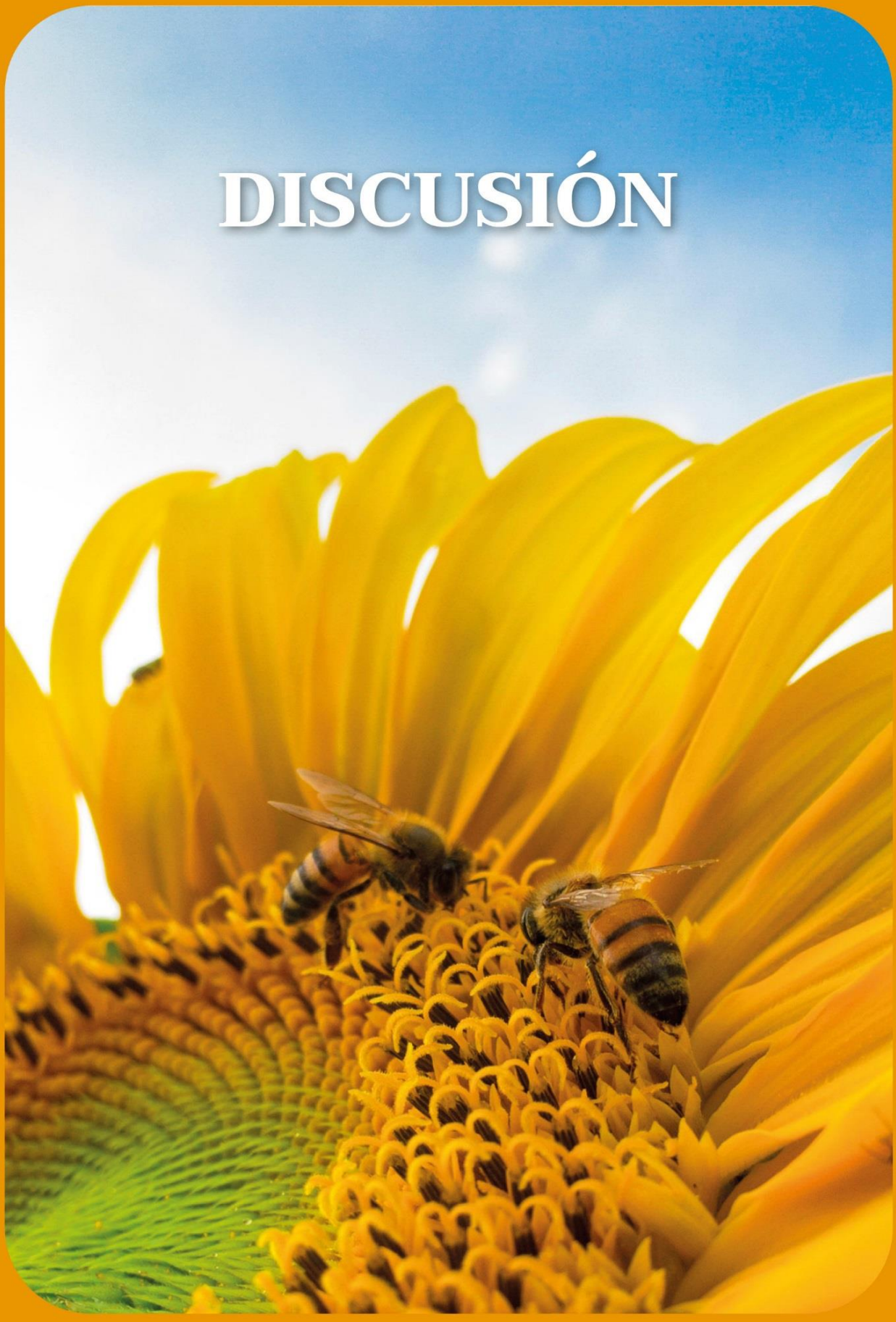
# DISCUSIÓN



# DISCUSIÓN

Las abejas continúan desapareciendo. Cada año, millones de estos insectos mueren y, en consecuencia, cesa su labor polinizadora. Las pérdidas en las poblaciones de estos insectos han sido descritas desde la antigüedad. Sin embargo, actualmente, el problema ha alcanzado las connotaciones de crisis. Sólo en España, la extinción de las abejas conllevaría el colapso de varios de los cultivos más importantes dentro del sector agrícola. El valor económico que representa la polinización por parte de las abejas alcanza cifras de aproximadamente 2.400 millones de euros en España, y hasta 265.000 millones en todo el mundo. Además, la polinización contribuye positivamente a la ecología del planeta, ya que favorece el mantenimiento de la biodiversidad, por lo que su decrecimiento afectaría de manera acusada al medio ambiente (vanEngelsdorp, Evans, et al., 2009).

Son varios los fenómenos implicados en esta problemática. Por un lado, la mortalidad invernal consiste en la muerte de la colmena tras la invernada. Las abejas no cuentan con suficientes reservas de alimento y cría y, por tanto, no son capaces de sobrevivir al invierno. Para que los niveles de mortalidad invernal sean tolerables por el colmenar, éstos deben mantenerse en torno al 5-10%, con el fin de no ocasionar pérdidas demasiado elevadas. Sin embargo, en los últimos años, se ha registrado un aumento de las mismas, llegando a niveles cercanos al 20% en algunos países, según los resultados preliminares del proyecto internacional COLOSS (Prevention of honey bee COlony LOSSes), sobre las pérdidas de colonias durante el 2017-18, presentado por la entidad Honey Bee Research Association (Genersch et al., 2010).

Por otro lado, desde las décadas de los 80-90, el SDC ha favorecido notoriamente el descenso en las poblaciones de abejas en todo el mundo (vanEngelsdorp, Evans, et al., 2009). Este fenómeno, que ya ha sido descrito en capítulos previos, ha cobrado gran importancia para la comunidad científica. Se ha convertido, de hecho, en uno de los principales temas de interés para la investigación. Tanto es así que la mayor parte de los países han iniciado programas de vigilancia de pérdidas en sus poblaciones para cuantificar y valorar la gravedad de las mismas. A

su vez, se han desarrollado nuevos métodos de monitoreo de las colmenas y se ha enaltecido la necesidad de estudiar a fondo cada uno de los factores implicados tanto en el SDC como en las pérdidas invernales, dado el carácter multifactorial de ambos problemas (vanEngelsdorp, Evans, et al., 2009).

Numerosas causas han sido identificadas como potenciales desencadenantes de estos síndromes: pesticidas, deficiencias nutricionales, agentes patógenos y parásitos, el cambio climático, mal manejo por parte del apicultor, etc. Es importante tener en cuenta la condición de súper organismo que caracteriza a las colmenas, de tal modo que distintos factores pueden actuar a nivel individual y repercutir, sin embargo, en la respuesta inmune de la colmena, haciéndola más susceptible a las enfermedades. Como ha sido apuntado con anterioridad, en la colmena se establece un equilibrio fundamentado en tres pilares básicos: la abeja (el hospedador), el medio ambiente tanto interno (la colmena) como externo (el clima y el hábitat del colmenar, entre otros), y los agentes patógenos capaces de producir enfermedad en las abejas. Las bacterias, los virus, los hongos y los parásitos van a encontrarse siempre, en mayor o menor medida, afectando a los individuos de la colmena. Niveles tolerables de éstos no tendrán repercusiones en la colmena, ya que las abejas cumplirán sus funciones sin impedimento y, en el caso de haber bajas, los individuos emergentes reemplazarán a los que han muerto. Sin embargo, cuando este equilibrio entre el hospedador, el medio ambiente y los patógenos se rompe, éstos últimos empiezan a proliferar a niveles mayores de los asumibles por la colmena, de tal modo que la reposición de abejas no es capaz de suplir las necesidades productivas de la colmena. Un buen ejemplo de esta situación es el caso de los virus y el ácaro *Varroa destructor*. Los virus suelen estar presentes en las colmenas produciendo infecciones persistentes encubiertas (Chen & Siede, 2007). A estos niveles de replicación, las consecuencias de la infección incluyen cierto desgaste energético a largo plazo y un leve descenso de la capacidad productiva del individuo. No obstante, la colmena es capaz de compensar las pérdidas. Sin embargo, cuando el ácaro se introduce en la

colmena y comienza su ciclo reproductivo, su tasa reproductiva aumenta, muchas veces de manera semejante a la de la propia colmena (Francis et al., 2013). La varroa se alimenta del cuerpo graso y de la hemolinfa de las abejas emergentes y continúa parasitando a las abejas adultas, de tal modo que las abejas que nacen son más débiles y muchas de ellas presentan una vida productiva mucho menor (Guzmán-Novoa et al., 2010; Nazzi et al., 2012; Ramsey et al., 2019). Por tanto, la presencia de varroa en la colmena conlleva debilitamiento de la misma. A su vez, el ácaro es capaz de transmitir numerosos virus a las abejas, como por ejemplo DWV, IAPV o ABPV (Di Prisco et al., 2016; Francis et al., 2013). La transmisión de dichos virus por parte del ácaro tiene una serie de repercusiones: la varroa inyecta los virus directamente en la hemolinfa de las abejas, lo que favorece el ciclo de replicación de los mismos (Francis et al., 2013); a su vez, las abejas se encuentran más débiles como consecuencia de la parasitación y, por tanto, los virus, que actúan como patógenos oportunistas, encuentran en la colmena las condiciones idóneas para su replicación. Si además existen deficiencias nutricionales u otras alteraciones relacionadas con el medio ambiente de la colmena, las infecciones víricas proliferan y la parasitación por varroa continúa debilitando a las abejas, lo que ocasiona la muerte de un gran número de individuos. En este caso, la colmena no puede compensar las pérdidas y colapsa en la mayoría de las ocasiones.

Dadas todas estas premisas, el enfoque empleado a lo largo de esta tesis doctoral ha tenido en cuenta los diversos factores implicados en la problemática que actualmente afecta a las abejas, centrándose en los pilares esenciales que sustentan el equilibrio dentro de la colmena. En primer lugar, se ha estudiado la secuencia del virus DWV para elucidar su relación con la virulencia del mismo y establecer así medidas de control diferentes en función de la variante presente en la colmena (objetivo 1). En segundo lugar, se han analizado distintos genes implicados en el sistema inmunitario de las abejas para desarrollar potenciales marcadores de sistema inmunitario que permitan monitorizar el estado de salud de las colmenas (objetivo 2).

En tercer lugar, se ha estudiado la relación entre los agentes patógenos y la nutrición de la colmena, teniendo en cuenta para ello la ingesta de polen (sub objetivo 3.1). A su vez, se han evaluado las localizaciones de los apiarios con el fin de estudiar las repercusiones del medio ambiente sobre la salud de las colmenas (sub objetivo 3.2). Por último, se ha explorado la relación entre los parámetros indicadores de fortaleza de las colmenas y la carga de los virus y el parásito *Varroa destructor* (objetivo 4). Para llevar a cabo todos estos objetivos, se contó con dos escenarios distintos. Por un lado, los objetivos 1, 2 y 4 se realizaron a partir de las muestras obtenidas del colmenar experimental situado en el Centro Apícola de la Universidad de Córdoba. Este colmenar, que contaba con 10 colmenas Langstroth, fue muestreado mensualmente desde marzo de 2015 a enero de 2017. Por otro lado, se contó con ocho colmenares situados en la isla de Tenerife, que fueron empleados para el desarrollo del objetivo 3 de esta tesis doctoral.

A pesar de que se han estudiado diversos virus que afectan a las abejas, esta tesis doctoral se ha centrado en el virus de las alas deformadas y su relación con el ácaro *Varroa destructor*. Numerosos estudios han corroborado la importancia de estos dos patógenos en el desencadenamiento de pérdidas en las colmenas. Tanto es así, que se han postulado como marcadores predictivos de las pérdidas en las colmenas (Dainat et al., 2012b; Kielmanowicz et al., 2015). Se han abordado, en primer lugar, los aspectos relativos al virus (variaciones genéticas del virus y el papel del sistema inmunitario de las abejas en presencia del mismo), para posteriormente estudiar en mayor profundidad su relación con el ácaro *Varroa destructor*, la nutrición de las abejas y el desarrollo de sintomatología/mortalidad en las colmenas, teniendo en cuenta las condiciones de producción y medio ambientales de distintas regiones de España.

Como ha sido discutido en los puntos anteriores, el virus DWV es, actualmente, una de las mayores amenazas para el sector apícola junto con el ácaro *Varroa destructor.* Su implicación en la mortalidad de las colmenas ha sido

ampliamente estudiada (Dainat et al., 2012b; Dainat & Neumann, 2013; F. Nazzi & F. Pennacchio, 2018; Ravoet et al., 2013). En los últimos años se han llevado a cabo importantes avances en el conocimiento de este virus: gracias a herramientas como la metagenómica y la secuenciación masiva, se ha podido analizar su secuencia, lo que ha contribuido a una mejor comprensión de la patogenicidad del virus. Sin embargo, desde la propagación masiva de la varroa por todo el mundo, el virus ha sufrido una serie de modificaciones genéticas que han dado lugar a cambios en su patogenicidad y virulencia. Diversos investigadores han descubierto la existencia de nuevas variantes de DWV: por un lado, encontramos Kakugo virus (KV), que es clasificado como la variante japonesa del DWV original denominada DWV-A, y, por el otro lado, *Varroa destructor* virus (VDV-1), también llamado DWV-B. En 2016, Mordecai y colaboradores evidenciaron la existencia de una tercera variante de DWV, a la que denominaron DWV-C y que comparte un 79,1% de amino ácidos comunes con DWV-A y un 78,9% con DWV-B (Mordecai, Wilfert, et al., 2016). Dichos porcentajes de disimilitud son suficientes para considerar la secuencia como una nueva variante, de acuerdo a los criterios establecidos (Fauquet & Fargette, 2005; Van Regenmortel et al., 1997)

La replicación del virus dentro del ácaro *Varroa destructor* conlleva una reducción drástica en la diversidad genética del virus, lo que promueve la selección de una u otra variante genética (Mordecai, Brettell, et al., 2016; Mordecai, Wilfert, et al., 2016). En consecuencia, la presencia de la varroa en una colmena puede llegar a ser determinante para que se produzca el desarrollo de la infección por DWV. Como ha sido descrito en el capítulo uno de esta tesis doctoral, la virulencia del virus puede variar en función de la variante, de tal modo que se ha postulado que DWV-A y KV son más virulentos que DWV-B/VDV-1. En el estudio de Mordecai y colaboradores (Mordecai, Brettell, et al., 2016) propusieron la existencia del fenómeno denominado "exclusión por superinfección" en colmenas infectadas por varroa, donde la abundancia de DWV-B parecía tener un efecto protector sobre las colmenas. Según

esta hipótesis, la selección de dicha variante evitaría la replicación de DWV-A (supuestamente más virulenta), favoreciendo la supervivencia de la colmena.

Hasta el momento, en España no se había realizado un estudio para determinar las variantes de DWV presentes y sus implicaciones en la mortalidad de las colmenas. Para dar respuesta a estas incógnitas y completar el primer objetivo de esta tesis doctoral, se llevaron a cabo los experimentos descritos en el primer capítulo. Para ello, se seleccionaron dos colmenas procedentes del colmenar experimental situado en la Universidad de Córdoba y se realizó un seguimiento de las mismas. La primera de ellas, denominada Colmena H (de las siglas en inglés, Healthy), sobrevivió a lo largo de todo el periodo de estudio, mostrando un estado de salud aceptable en las distintas evaluaciones mensuales. Los niveles de varroa y DWV fueron elevados en la mayor parte de los muestreos, mostrando disminuciones tras el tratamiento acaricida. Por otro lado, la segunda colmena, denominada Colmena W (de las siglas en inglés, Weak), colapsó antes de finalizar el periodo de estudio, mostrando sintomatología tal como mortalidad, abejas con deformidad en las alas y escasez de población. Los niveles de DWV y varroa en esta colmena también fueron elevados, de modo similar a la Colmena H. Dado que la evolución de la carga de patógenos fue bastante similar en ambas colmenas, pese a que la respuesta a la infección y a la parasitación fue diferente, se decidió evaluar la secuencia del virus en ambas colmenas. De este modo, se seleccionó una muestra positiva a DWV y con carga elevada en cada colmena para su estudio mediante secuenciación. Para mejorar los análisis de secuenciación, se aplicó la técnica "Whole transcriptome amplification" en el procesado de ambas muestras. Este novedoso protocolo permite la síntesis de ADN complementario (cADN) a partir de ARN, maximizando la cantidad de material genético generado y la calidad de mismo, lo que favorece unos mejores resultados en la secuenciación posterior de las muestras y, por ende, en el análisis filogenético de las mismas. Tras este procedimiento, se aplicó el enfoque "primer walking" para la obtención de la secuencia completa de ambas muestras, que consiste

en la división en fragmentos más pequeños de una secuencia demasiado larga para su secuenciación directa (França, Carrilho, & Kist, 2002). Tan sólo una pequeña proporción de los nucleótidos de los extremos de la secuencia (correspondientes a parte no codificante del genoma) quedaron sin secuenciar.

El estudio en profundidad de las secuencias reveló diferencias significativas entre las dos muestras, que compartieron un 85,1% de los nucleótidos. Cuando ambas secuencias se compararon con aquellas de referencia para DWV-A y DWV-B, la muestra procedente de la Colmena H fue clasificada como recombinante entre DWV-A y DWV-B, más similar a DWV-B, mientras que la muestra procedente de la Colmena W fue clasificada como DWV-A. Se llevó a cabo la construcción de dos árboles filogenéticos. El primero de ellos se realizó con las secuencias completas de DWV-A y DWV-B disponibles en el GenBank, de dónde además se extrajo información acerca de la salud de las colmenas correspondientes. El segundo árbol se construyó utilizando multitud de secuencias de la proteína polimerasa del genoma de DWV, región muy variable y que permitió corroborar los resultados obtenidos en el análisis previo. Ambos árboles evidenciaron la similitud de la secuencia de la Colmena H con la secuencia de DWV-B y de la secuencia de la Colmena W con la secuencia de DWV-A y KV. Teniendo en cuenta la evolución del estado de salud de ambas colmenas, se postuló que las diferencias en nucleótidos podrían tener grandes repercusiones en la virulencia del virus, de tal modo que la dominancia de la variante DWV-A estaría relacionada con la mortalidad de las colmenas y la de la variante DWV-B con la supervivencia de éstas.

Dado que los resultados habían sido obtenidos en tan sólo dos colmenas, se decidió corroborar la hipótesis en un mayor número de muestras. Para ello, se aplicó el análisis ABC desarrollado por Kevill y colaboradores (Kevill et al., 2017), que permite la diferenciación de las tres variantes de DWV mediante RT-qPCR. Los resultados obtenidos tanto en el resto de colmenas como en el seguimiento de las colmenas H y W revelaron una mayor presencia de DWV-A en aquellas colmenas con

sintomatología, peor estado sanitario o mortalidad, mientras que DWV-B fue más abundante en las colmenas sanas. Estos resultados apoyan la hipótesis inicialmente planteada y exaltan la importancia de evaluar la presencia de las distintas variantes de DWV, en tanto que la dominancia de una u otra puede resultar determinante para la colmena. Así pues, la secuenciación puntual de muestras positivas a DWV en los periodos de mayor riesgo de pérdidas podría aportar información relevante acerca del estado de salud de las colmenas. Por ejemplo, si la variante DWV-A es la más presente en la colmena, se deberían tomar medidas de control urgentes, tales como la aplicación de tratamientos acaricidas para eliminar la varroa (transmisor del virus) o alimentación suplementaria que favorezca el sistema inmunitario de las abejas y, con ello, ayude a la colmena a hacer frente a la infección.

No se detectó DWV-C en ninguna de las muestras analizadas, lo que podría significar que no se trata de una variante ampliamente establecida en el sur de España por el momento. Sin embargo, al tratarse de un estudio llevado a cabo en un solo colmenar, estos resultados deben ser tomados con precaución. Futuros estudios, por tanto, deberían evaluar la presencia o ausencia de la variante DWV-C en el país, que sí ha sido detectada en otros países de Europa y en Estados Unidos (Mordecai, Wilfert, et al., 2016; Natsopoulou et al., 2017; Ryabov et al., 2014).

Sin embargo, la diversidad genética del virus no es el único factor implicado en la respuesta de la colmena a la infección. Como ha sido mencionado previamente, el sistema inmunitario de las abejas cumple un papel clave para que el equilibro dentro de la colmena se mantenga y, por consiguiente, las abejas sean capaces de lidiar con la patogenicidad derivada de DWV y *Varroa destructor*. Es por ello que el estudio del sistema inmunitario, tanto a nivel individual como de súper organismo, ha sido uno de los aspectos más investigados en los últimos años (Evans, 2004; Huang et al., 2012; Steinmann et al., 2015). El incremento de las pérdidas y la aparición de nuevos patógenos con elevada virulencia, ha suscitado las siguientes cuestiones: ¿qué mecanismos de defensa emplean las abejas frente a los distintos factores

involucrados en estas pérdidas? ¿existen mecanismos específicos para la defensa de patógenos como los virus o varroa? ¿podría estimularse de manera externa la respuesta inmune de las abejas, para así prevenir pérdidas?

Hasta la fecha, poco se conoce del sistema inmunitario de las abejas. Al igual que el resto de insectos, las abejas carecen de inmunoglobulinas y de respuestas adaptativas, ya que no cuentan con memoria inmunológica. Debido a esto, no es posible la prevención de enfermedades mediante la vacunación. Evans y colaboradores (Evans et al., 2006) evidenciaron, además, que *Apis mellifera* cuenta con una respuesta inmunitaria individual menos eficiente que otras especies de insectos. Este hecho se ve compensado, sin embargo, por la existencia de una respuesta inmune colectiva muy optimizada (Hillyer, 2016).

Cuando un organismo patógeno ataca a la abeja, se encuentra con tres líneas defensivas. La primera línea de defensa son las barreras mecánicas del tegumento (cutícula, exoesqueleto) y de los tejidos epiteliales internos. Estos mecanismos actúan como barrera para los patógenos. Sin embargo, cuando el agente logra atravesar la primera línea de defensa, se produce la respuesta celular, basada en la presencia de los hemocitos en la hemolinfa. Éstos, se encargan de fagocitar cuerpos extraños, de la aglutinación y de la producción de radicales intermediarios de oxígeno (ROI) y de nitrógeno (RNI), con potente acción microbicida. Por último, si los patógenos logran escapar de la respuesta celular y penetrar en las células, tiene lugar la respuesta humoral, fundamentada en la producción de moléculas intracelulares (péptidos antimicrobianos) (McMenamin et al., 2018). Esta respuesta es, por tanto, decisiva, ya que es la última barrera frente a los patógenos.

La respuesta inmune humoral está formada, a su vez, por los siguientes sistemas (Evans et al., 2006):

1) Péptidos y proteínas antimicrobianas.
2) Sistema de la fenoloxidasa.

3) Cascadas enzimáticas que regulan la melanización y coagulación de la hemolinfa.

Debido a su importancia en la defensa frente a los patógenos, el segundo objetivo de esta tesis doctoral, descrito en el capítulo dos, se ha centrado en el estudio de la respuesta inmune de base humoral. Se ha empleado para ello un enfoque genómico, dado que la base de este tipo de respuesta inmune se fundamenta en la información almacenada en los genes, cuya expresión regula la activación de las distintas vías inmunes.

Dentro de dicha respuesta humoral, las abejas cuentan con una serie de vías inmunes innatas (Toll, Imd, JAK/STAT y JNK) que se encargan de activar la melanización (mediante el sistema feniloxidasa), producir efectores antimicrobianos (péptidos como *defensin, abaecin* o *apidaecin*) y llevar a cabo la degradación dependiente del proteosoma (Evans et al., 2006). Para comprender mejor el funcionamiento de estas vías, se estudiaron cuatro genes involucrados en las mismas como parte del objetivo 2 de esta tesis doctoral: *relish* (vía Imd), *defensin* (péptido antimicrobiano), *dorsal* (vía Toll) y *domless* (vía JAK/STAT). El principal objetivo de este estudio fue desarrollar marcadores basados en los genes analizados que nos permitiesen identificar situaciones de riesgo en la colmena. Para ello, se seleccionaron siete de las diez colmenas correspondientes al colmenar experimental de la Universidad de Córdoba y se llevó a cabo un seguimiento mensual de cada una de ellas durante un periodo de 12 meses. Se analizó la carga de dos virus (DWV y BQCV) y la infestación por el parásito *Varroa destructor.* Se procedió también a la evaluación de los cuatro genes del sistema inmunitario seleccionados (*relish, defensin, dorsal* y *domless*).

Por un lado, el análisis estadístico de los resultados evidenció una correlación positiva entre la expresión de *relish* y la carga de DWV y varroa, de tal modo que el incremento en la expresión de este gen podría reflejar un aumento en la carga de estos dos patógenos. Por otro lado, se observó una correlación negativa entre la

expresión de *defensin* y los niveles del binomio DWV-varroa, lo que podría implicar que ambos agentes actúan inhibiendo la producción de este péptido antimicrobiano. Además, aquellas colmenas que colapsaron durante el estudio presentaron regulación negativa de este gen en los meses previos al colapso, mientras que el gen se expresó en mayor proporción en aquellas colmenas que sobrevivieron a pesar de las altas cargas de DWV y varroa. Aunando estos resultados, la valoración conjunta de estos dos genes del sistema inmunitario (*relish* y *defensin*) podría ser utilizada como marcador del riesgo en la colmena: altos niveles de *relish* reflejarían el incremento de la carga de DWV y varroa, que, combinado con una regulación negativa de *defensin* mostraría la incapacidad de la colmena para hacer frente a la infección. De este modo, se podrían tomar medidas preventivas en la colmena afectada y evitar así su colapso.

Se evidenció también una correlación negativa entre la expresión del gen *dorsal* y la carga de DWV. Esta relación había sido previamente descrita (Khongphinitbunjong et al., 2015; Van Rij et al., 2006) y podría reflejar una relación directa entre la vía inmunitaria Toll y la respuesta frente a la infección por DWV. Además, se observó que incrementos en la expresión de *dorsal* conllevaron un descenso en la carga de DWV, por lo que futuros estudios deberían explorar la capacidad de este gen para controlar la infección por el virus. *Dorsal* también estuvo correlacionado negativamente con la carga de BQCV, lo que apoya la teoría de que la vía Toll puede verse suprimida en presencia de los virus.

Adicionalmente, se estudió la evolución de la expresión de cada gen temporalmente, teniendo en cuenta las diferentes estaciones. Así, se observaron niveles mayores de expresión durante la primavera y el verano, con excepción del gen *defensin*, cuyo pico se alcanzó durante el invierno, coincidiendo con niveles de DWV y varroa más bajos.

Los resultados de este objetivo, por tanto, han supuesto un gran avance en el estudio del sistema inmunitario de las abejas, aportando evidencias del papel de los

distintos genes analizados. El sistema inmunitario de las colmenas es, por tanto, un factor clave, como ha sido evidenciado a lo largo del segundo capítulo de esta tesis doctoral, por lo que su evaluación resulta fundamental a la hora de detectar problemas de manera temprana en la colmena. El empleo de *relish* y *defensin* como marcadores del estado sanitario de las colmenas aportaría valiosa información para el apicultor. Midiendo estos genes en los periodos de mayor riesgo (tras la invernada, en épocas de climatología adversa o cuando la infestación por varroa es más elevada, por ejemplo) podríamos realizar una aproximación al estado de salud de la colmena: niveles elevados de *relish* en combinación con regulación negativa de *defensin* podrían ser indicativos de que la infección por DWV y la infestación por varroa son potencialmente elevadas y que la colmena no se encuentra en condiciones de afrontarlas. Si esta evaluación se complementase también con la secuenciación del virus para determinar la variante presente en la colmena (basándonos en los resultados obtenidos en el primer objetivo de esta tesis doctoral), se alcanzaría un conocimiento de gran profundidad acerca del estado de salud de la colmena. Todo ello facilitaría la toma de decisiones y permitiría al apicultor y al veterinario apícola llevar a cabo las acciones pertinentes para evitar el colapso de la colmena.

No obstante, el estudio de la salud de las abejas no puede ser abordado únicamente desde una perspectiva molecular. Como ha sido apuntado a lo largo de la presente tesis doctoral, la supervivencia de la colmena se fundamenta en el mantenimiento de un equilibro muy vulnerable, donde los patógenos y el sistema inmunitario tienen un gran peso. Sin embargo, no son los únicos factores determinantes. El medio ambiente, tanto externo como de la propia colmena, es también un pilar básico que contribuye al adecuado balance dentro de la colmena (Asensio et al., 2016; Gallant et al., 2014; Klein et al., 2007). La relación que se establece entre el medio ambiente y las abejas adquiere prácticamente las connotaciones de una simbiosis: estos insectos polinizan un gran número de flores, siendo imprescindibles para la agricultura y participando activamente en el

mantenimiento de la biodiversidad de gran parte del planeta. Otro factor clave es la nutrición de las abejas, que será determinante para el adecuado desarrollo de los individuos y su productividad. Estas cuestiones han sido abordadas en el tercer capítulo de esta tesis doctoral, correspondiente al objetivo 3, el cual se dividió en dos sub objetivos: por un lado, se estudió la relación entre la diversidad de polen recolectado por las abejas y la respuesta a las enfermedades víricas y el parásito *Varroa destructor*; por otro lado, se evaluaron las implicaciones de las características medio ambientales de los apiarios en la salud de las colmenas.

Para llevar a cabo dicho estudio se utilizó la isla de Tenerife como modelo, debido a sus características medio ambientales particulares y a que cuentan con una apicultura muy peculiar, basada en una producción artesanal y de gran tradición (Casa de la miel, 2014). La Isla de Tenerife cuenta con una serie de propiedades que favorecen la presencia de distintos microclimas, lo que aporta una gran riqueza para la producción apícola. Esto favorece también la aparición de una flora muy diversa y, por consiguiente, dota a la isla de una gran variedad de plantas disponibles para la obtención de polen por parte de la abeja. Dado que la diversidad de polen ha sido considerada como un factor que contribuye positivamente a la salud y producción de las colmenas (Di Pasquale et al., 2013), se planteó la siguiente hipótesis: si las abejas recolectan polen de mayor diversidad, esto podría verse reflejado en un mejor sistema inmunitario, lo que repercutiría en una mejor respuesta a las enfermedades y, en consecuencia, un mejor estado de salud de la colmena.

Para ello, se recogieron muestras de polen en ocho colmenares distintos localizados en la isla durante el periodo comprendido entre abril y septiembre de 2014. En cada colmenar, se realizaron hasta nueve muestreos de polen y, adicionalmente, se evaluó la fortaleza de las colmenas al inicio y al final del estudio. También se llevó a cabo un muestreo sanitario al final de la temporada para el análisis de virus y varroa. En primer lugar, se determinó el número de especies diferentes en una misma muestra de polen (riqueza del polen) en función de los colores de las

pelotas de polen recolectadas. Posteriormente, se efectuó el análisis palinológico del polen con el fin de profundizar en su examen y conocer el origen del mismo. En último lugar, se calculó el índice de Shannon para determinar la diversidad de cada muestra de polen.

Como el objetivo de este estudio era evaluar las implicaciones de la diversidad del polen en la capacidad de respuesta a las enfermedades, los datos de diversidad polínica se estudiaron conjuntamente con los datos obtenidos a partir de los análisis de patógenos y los datos epidemiológicos referidos al estado de salud de las colmenas (síntomas y mortalidad), ya que en capítulos previos se constató la relación entre DWV y varroa con la salud y el estado inmune de las colmenas. Los resultados obtenidos fueron un tanto inesperados, puesto que no se evidenció ninguna relación entre la diversidad de polen y el estado sanitario de las colmenas. Únicamente se evidenció una correlación positiva entre los niveles de varroa y una mayor diversidad de polen, aunque el coeficiente de correlación fue muy débil. Una posible explicación de este resultado es que una mayor diversidad de polen podría tener un efecto positivo en la colmena, tal y como se había postulado previamente. Sin embargo, en lugar de ejercer un efecto protector directo sobre la colmena, podría verse reflejado en un incremento de la cría, lo que, a su vez, favorecería la reproducción de la varroa (Evans & Cook, 2018).

A pesar de que el sub objetivo 3.1 se centró en el efecto del polen y la nutrición, no se observaron claras evidencias de su relación con la salud de las colmenas, al menos con la metodología empleada. Sin embargo, existen varios factores del diseño experimental de este estudio que deben ser tenidos en cuenta a la hora de interpretar los resultados. En primer lugar, los patógenos sólo fueron analizados al final del estudio y en un único muestreo, por lo que los análisis estadísticos se realizaron con un menor número de datos de lo que habría sido ideal. En segundo lugar, otra posible explicación de estos resultados es que la diversidad de polen podría no ser un factor determinante para la salud de las colmenas en las

condiciones particulares de Tenerife, en contraposición a otros estudios (C Alaux et al., 2010; Antunez et al., 2015; Di Pasquale et al., 2013). No obstante, los resultados obtenidos han abierto el camino a futuros estudios, ya que resultaría de interés evaluar la relación directa entre la diversidad del polen, así como su calidad, y el sistema inmunitario de las abejas. De este modo, se podrían evaluar las repercusiones de la dieta e identificar aquellos factores más influyentes en la salud de las abejas. Por otro lado, el análisis palinológico reveló que las muestras de polen se componían principalmente por especies catalogadas como flora de interés apícola (*Cistus monspeliensis, Kleinia nerifolia*, entre otras) (Kunkel, 1991; Santos, Bentabol, Hernández, & Modino, 2004), siendo algunas de ellas plantas endémicas en la isla. La diversidad del polen varió a lo largo de los meses, alcanzándose los valores más altos durante la primavera.

Como parte del mismo capítulo, además del estudio de la interacción de la salud de la colmena con la nutrición, se procedió a la evaluación de las condiciones medio ambientales de los colmenares. Un emplazamiento con condiciones adecuadas y que garantice el acceso a los recursos es fundamental para que la colmena lleve a cabo sus funciones. Por ello, la selección de los emplazamientos debe realizarse tras una evaluación previa, teniendo en cuenta distintos aspectos. Asensio y colaboradores (Asensio et al., 2016) determinaron la importancia de un gran número de componentes medio ambientales, utilizando la Comunidad de Madrid como modelo. Siguiendo su enfoque, y como parte del tercer objetivo de esta tesis doctoral (sub objetivo 3.2), se evaluó la calidad medio ambiental de los ocho colmenares estudiados en la Isla de Tenerife mediante el empleo de herramientas de visualización espacial: el mapa cartográfico de Tenerife (Grafcan, 2015), Corine Land Cover ("Corine Land Cover, seamless vector data (Version 17)," 2006) y ArcGIS (ESRI, 2011).

Así pues, se estudió la calidad del paisaje en función de seis factores: número de coberturas vegetales (F1), número de especies vegetales útiles para la abeja (F2), distancia a fuentes de agua corriente permanentes (F3), presencia de infraestructuras

de origen humano (F4), presencia de granjas de producción animal (F5) y presencia de superficies con cultivos (F6). Cada uno de estos factores fue evaluado y clasificado en dos categorías: a) condición adecuada para la producción apícola o b) condición inadecuada para la producción apícola. Cada colmenar fue clasificado, a su vez, en función de los resultados anteriores. El estudio individualizado de cada colmenar evidenció que aquellos emplazamientos en los que existían condiciones inadecuadas para la producción apícola mostraban mayores cargas de virus y varroa con respecto a los colmenares que contaban con buenas condiciones. Además, la presencia de sintomatología (deformidad en las alas, presencia de abejas muertas alrededor de la colmena, menor población, etc.) fue más abundante en colmenares donde las condiciones ambientales eran más pobres, basado en los factores definidos previamente. Estos resultados enfatizan la importancia de realizar un análisis ambiental de los emplazamientos apícolas, teniendo en cuenta factores tales como la heterogeneidad del paisaje, abundancia de floraciones adecuadas para las abejas, presencia o ausencia de infraestructuras que fragmenten el paisaje o cercanía a fuentes de agua permanentes, entre otros.

Por lo tanto, teniendo en cuenta los resultados descritos en el tercer capítulo de esta tesis doctoral, se ha podido constatar la importancia de elegir un emplazamiento adecuado para la instalación de los colmenares, aunque no se ha comprobado que una mayor diversidad de polen ejerza un efecto positivo en la respuesta de las abejas frente a las enfermedades, al menos con las condiciones de muestreo aplicadas y el diseño experimental propuesto.

Como ha sido constatado a lo largo de los capítulos previos, la búsqueda de soluciones prácticas a problemas comunes en la apicultura ha sido la premisa de esta tesis doctoral. Una vez abordados de manera individualizada aquellos factores más influyentes en la salud de la colmena, surgió la necesidad de congregar todos los resultados obtenidos y aportar herramientas útiles al apicultor, además de los marcadores de salud desarrollados (secuencia de DWV o genes del sistema inmune).

Así pues, en vista de los hallazgos derivados de los objetivos 1, 2 y 3, se planteó la posibilidad de estudiar la relación sinérgica existente entre DWV y varroa, así como su implicación en la salud de las colmenas, de un modo más extendido en el tiempo. Además, quisimos valorar la eficacia de la medición de la fortaleza de la colmena a través del vigor, parámetro basado en la opinión experta del apicultor o técnico apícola, en comparación con el método estandarizado (Delaplane et al., 2013b). Así pues, en el cuarto objetivo de esta tesis doctoral, que se corresponde con el cuarto capítulo de la misma, se realizó la evaluación de las 10 colmenas pertenecientes al colmenar experimental de la Universidad de Córdoba en un periodo de 21 meses. Como resultado de este trabajo, se generaron 142 muestras correspondientes a cada una de las colmenas a lo largo del tiempo. Mensualmente, se determinó la carga de DWV y varroa (y de los virus BQCV, SBV e IAPV, adicionalmente), así como la fortaleza de la colmena, que fue medida mediante dos métodos distintos: el método subjetivo estandarizado (basado en la cantidad de abejas con respecto a una población diana) y el vigor (basado en la opinión experta del técnico apícola y la observación de sintomatología). En primer lugar, se observó una tendencia clara de ambos agentes a aumentar sus cargas en los periodos de verano y otoño. La carga de DWV y varroa estuvo correlacionada tanto a nivel individual (colmenas) como a nivel global (colmenar). Además, se efectuaron tratamientos frente al ácaro que disminuyeron eficazmente la carga de DWV, por lo que se confirmó la existencia de una relación sinérgica entre ambos agentes (Di Prisco et al., 2016). El binomio DWV-varroa conlleva la desestabilización del delicado balance entre el sistema inmunitario y el virus, permitiendo el paso de infección encubierta a infección aparente (Di Prisco et al., 2016). Recientes estudios han apuntado que la relación sinérgica entre ambos agentes pueda tener repercusiones negativas en los miembros de la ruta NK-B, de tal modo que se produce la inmunosupresión de este mecanismo, permitiendo la proliferación del virus y el desarrollo de síntomas tales como deformidad de alas o mortalidad (Nazzi et al., 2012). Como se demostró en el objetivo 2 de esta tesis

doctoral, ambos agentes tienen la capacidad de modular también los genes implicados en las rutas Imd y Toll.

Como ha sido apuntado previamente, se evaluó la fortaleza de la colmena a lo largo del tiempo mediante dos métodos distintos, que mostraron una evidente correlación. Se estableció, así, que la valoración del vigor por parte de un técnico apícola experimentado tiene una gran validez a la hora de determinar la fortaleza de la colmena, dando resultados muy similares a los obtenidos mediante el método subjetivo establecido por el COLOSS (Delaplane et al., 2013b). El método del COLOSS se basa en la estimación visual del número de celdas ocupado por una población diana, valor que se transforma mediante una fórmula matemática y nos proporciona un valor de población (Delaplane et al., 2013b). A pesar de ser un método bastante preciso, la necesidad de abrir y extraer todos los cuadros de la colmena, así como el tiempo requerido para el conteo, hacen que este método no sea siempre el más factible. Por ejemplo, en colmenas débiles, la apertura y extracción de los cuadros durante un tiempo prolongado podría tener graves consecuencias en las abejas. El vigor, sin embargo, puede ser medido de manera más rápida y sin necesidad de estimar detalladamente la población en todos los cuadros, basándose en puntos clave como la actividad de las abejas y el espacio entre cuadros. Por lo tanto, el empleo del vigor como método de estimación de la fortaleza presenta ciertas ventajas: se trata de una técnica menos invasiva y más rápida; puede llevarse a cabo fácilmente por el apicultor experimentado o el técnico apícola, siendo muy útil durante la realización de experimentos; y no precisa la conversión de los datos. Los resultados de este objetivo, por tanto, han supuesto una gran mejora en el diseño y consecución de futuros experimentos, ya que el uso del vigor como parámetro para medir la fortaleza de la colmena facilitaría la evaluación de las mismas y agilizaría el proceso.

Mediante un modelo lineal mixto generalizado, se estudió la relación entre el vigor de las colmenas y la evolución de la infección por DWV y de la infestación por el ácaro a lo largo del tiempo. Se observó una relación directa entre altas cargas de

ambos agentes y un menor vigor de las colmenas. De este modo, se ha comprobado de nuevo el valor de estos dos agentes como indicadores del estado de salud de las colmenas de *Apis mellifera.* La evaluación periódica de estos dos patógenos en las colmenas puede ser orientativa para determinar el estado de salud de las mismas, incluso antes de que pueda ser percibido por el apicultor. Por ejemplo, un aumento marcado de la carga de DWV y varroa en una colmena determinada puede alertar al apicultor de que dicha colmena se encuentra en riesgo y que, por lo tanto, se deberían llevar a cabo medidas de control y prevención que ayuden a fortalecer la colmena (tratamiento frente a varroa, alimentación suplementaria, etc.).

Dado que parte de las colmenas colapsaron durante el periodo de estudio, y habiendo sido establecida la relación directa entre el binomio DWV/varroa y el vigor, se quiso explorar el efecto de los éstos en la supervivencia de las colmenas a lo largo del tiempo. Para ello, se llevó a cabo un estudio de supervivencia mediante el análisis de regresión de Cox (Lee, Wei, Amato, & Leurgans, 1992). Este método permite evaluar el riesgo de muerte de un sujeto a lo largo del tiempo en función de distintas variables. Las variables utilizadas fueron la carga de DWV y los niveles de infestación por varroa. El análisis reveló que el riesgo de muerte aumentaba con el incremento de los niveles de patógenos y que cuando la carga de éstos era elevada de manera mantenida en el tiempo, el periodo de supervivencia de la colmena solía oscilar en torno a los cuatro meses. Por lo tanto, resulta de gran importancia realizar un seguimiento periódico de las colmenas, determinando los niveles de DWV y varroa, para establecer medidas en función de los mismos. Si además se procediese a la secuenciación del virus para determinar la variante presente, la valoración del estado de salud y el riesgo de la colmena sería aún más precisa, en vista de los resultados obtenidos en el primer capítulo de esta tesis doctoral. De manera complementaria. También se podría ampliar la información obtenida mediante el empleo de los marcadores inmunológicos desarrollados en el segundo capítulo de esta tesis doctoral. La elección

de una metodología u otra dependerá de la disponibilidad de recursos y el nivel de detalle requerido.

Estos resultados también se observaron a lo largo del tercer capítulo de esta tesis doctoral. Como parte del estudio de la relación entre nutrición, medio ambiente y salud de las colmenas, se evaluó la presencia y carga de DWV y varroa en distintas colmenas de la isla de Tenerife (Islas Canarias). En dicha isla encontramos una raza autóctona, la abeja negra canaria. Esta raza cuenta con una serie de peculiaridades: se trata de una abeja menos agresiva (lo que facilita su manejo y ha contribuido al mantenimiento de la raza por parte de los apicultores), se caracteriza por ser de un color más oscuro y se encuentra completamente adaptada a las condiciones climatológicas de la isla. Por tanto, evaluar el papel de varroa y DWV en la abeja autóctona ha aportado datos de interés. Para ello, se evaluaron tres colmenas por emplazamiento (ocho colmenares en total). Se seleccionaron colmenas fuertes para que las condiciones de partida fuesen similares. En cada colmena, además del estudio del polen y del medio ambiente, se analizaron cuatro virus (DWV, BQCV, IAPV y SBV) y el ácaro varroa, así como la fortaleza de la colmena, al final del estudio, con el objetivo de conocer la evolución de las colmenas en paralelo al estudio del polen. Se llevó a cabo un análisis de regresión que reveló una relación entre DWV y varroa y la fortaleza de las colmenas, de tal modo que cargas altas de estos dos patógenos explicaban el desarrollo de sintomatología o descenso de la fortaleza (medido como pérdidas en la población, presencia de mortalidad en la colmena o síntomas evidentes). De este modo, se confirmaron los resultados obtenidos en el colmenar experimental del centro apícola de la Universidad de Córdoba también en la isla de Tenerife, evidenciando la necesidad de controlar los niveles tanto de DWV como de la varroa para favorecer el buen estado de salud de las abejas.

Considerando todos los resultados obtenidos a lo largo de esta tesis doctoral, se puede concluir que el estudio y la monitorización de las colmenas debe enfocarse desde una perspectiva holística, teniendo en cuenta el entorno de las mismas y

llevando a cabo análisis sanitarios regulares, con el objetivo de prevenir un aumento significativo de los patógenos, principalmente DWV y *Varroa destructor*. Ambos patógenos parecen ser decisivos para la supervivencia de la colmena, como ha sido demostrado en todos los capítulos de esta tesis doctoral y, especialmente, en el último capítulo de la misma (objetivo 4).

A su vez, el análisis filogenético de DWV ha permitido comprobar la existencia de al menos dos variantes de DWV en España, siendo, por lo tanto, un punto de partida para estudios posteriores. La relación entre la secuencia del virus y su virulencia ha sido demostrada en el primer capítulo de esta tesis doctoral, aunque convendría corroborar dichos resultados en un mayor número de muestras y en otras localizaciones. Además, sería interesante llevar a cabo un estudio para determinar la prevalencia de las distintas variantes en España, así como explorar la posible presencia de DWV-C en los territorios españoles.

Otro resultado de esta tesis doctoral ha sido el desarrollo de marcadores basados en la expresión de genes del sistema inmunitario, que ha supuesto un gran avance para la monitorización de las colmenas. Futuros estudios deberían explorar los resultados obtenidos en esta tesis doctoral y validar la eficacia de dichos marcadores en otras regiones de España y con otras condiciones de producción.

Dada la importancia del medio ambiente para las abejas, los emplazamientos para los colmenares deben ser elegidos en base a determinados criterios. A este respecto, se está iniciando un proyecto que tiene por objetivo la evaluación ambiental de distintas comunidades autónomas españolas con el fin de elaborar mapas de idoneidad para la instauración de explotaciones apícolas. La determinación de las condiciones ambientales de los diferentes emplazamientos supondrá, por tanto, una mejora sustancial para la apicultura. En este sentido, se han iniciado también estudios que tienen por objetivo el establecimiento de equipos de monitorización de las colmenas. Estos dispositivos, basados en sensores para distintas variables

(temperatura de la cría, peso de la colmena, sonido, horas de luz...) y en tecnología de visionado, permitirán conocer en profundidad las variaciones dentro de la colmena.

Sin embargo, y pese a que los objetivos de esta tesis doctoral han sido ambiciosos, aún quedan muchas incógnitas que resolver. La problemática actual de las abejas es un tema de preocupación social que genera graves repercusiones tanto económicas como medio ambientales, por lo que la comunidad científica debe considerar la prevención de las pérdidas de las poblaciones de abejas como uno de sus principales objetivos.

# CONCLUSIONES

# CONCLUSIONES

## PRIMERA / FIRST

Se han identificado dos variantes del virus de las alas deformadas en el sur de España: variante DWV-A y variante DWV-B, con recombinaciones frecuentes entre ambas. Las diferencias moleculares a nivel de secuencia del virus podrían estar relacionada con la virulencia del mismo, ya que la variante DWV-A fue encontrada en colmenas con un peor estado sanitario, mientras que DWV-B se relacionó con aquellas colmenas con mejor respuesta a la infección.

*At least two variants of the deformed wing virus have been identified in southern Spain, DWV-A and DWV-B, with frequent recombination between both variants. Molecular differences in the virus sequence may determine viral virulence. DWV-A was related to colonies with worse health, while DWV-B was related to colonies with better response to the infection.*

## SEGUNDA / SECOND

Los genes del sistema inmunitario *relish* y *defensin* pueden ser utilizados como marcadores del estado de salud de las colmenas. Mientras que altos niveles de expresión del gen *relish* son indicadores de cargas elevadas del virus de las alas deformadas y alto nivel de infestación por *Varroa destructor*, la regulación negativa del gen *defensin* reflejaría la incapacidad de la colmena para responder a la infección por ambos agentes.

Relish *and* defensin *are immune related genes that may serve as indicators of colony health. High* relish *expression may reflect increases in DWV and varroa levels, while negative regulation of* defensin *expression may serve as an indicator of how well a colony is likely to resist an existing infection of DVW*-Varroa destructor.

## TERCERA / THIRD

El gen del sistema inmunitario *dorsal* presenta regulación negativa en respuesta a la infección por el virus de las alas deformadas y el virus de las realeras negras, por lo que la ruta Toll del sistema inmunitario de las abejas podría tener un papel clave en la respuesta a la infección por dichos virus.

*The immune gen* dorsal *shows negative regulation in response to the infection by deformed wing virus and black queen cell virus. The Toll pathway may play a key role in the infection by both viruses.*

## CUARTA / FORTH

Paisajes heterogéneos, sin estructuras que lo fragmenten, con diferencias de altitud que permitan una mayor riqueza floral, así como cercanía a fuentes de agua o un gran número de coberturas vegetales están asociados con un mejor estado sanitario de la colmena.

*Heterogenous and unfragmented landscapes with difference in altitude and more diverse flora, as well as water resources and a great number of vegetation coverage are associated with better colony health.*

## QUINTA / FIFTH

La evaluación de los colores de las pelotas de polen puede ser una aproximación para la evaluación de la riqueza del polen, ya que los resultados obtenidos en cuanto al número de especies presentes en la muestra fueron muy similares a los obtenidos mediante el análisis palinológico. Sin embargo, la diversidad del polen no parece ser un factor fundamental a la hora de determinar el estado de salud de las colmenas, al menos en las condiciones particulares de la

apicultura en la isla de Tenerife (Islas Canarias, España) y con el diseño experimental llevado a cabo.

*Pollen pellets evaluation may be useful for pollen richness determination, since the palinological analysis revealed similar results. However, pollen diversity does not seem to be a key factor in ensuring colony health, at least in the particular beekeeping in Tenerife Island (Canary Island, Spain) and the experimental design used.*

## SEXTA /SIXTH

El virus de las alas deformadas y el ácaro *Varroa destructor* se encuentran altamente correlacionados. Entre ambos se establece una relación sinérgica que puede predisponer a la colmena a sufrir pérdidas e incluso provocar el colapso de la misma. El control del ácaro mediante la aplicación de tratamientos acaricidas efectivos contribuye también al control del virus de las alas deformadas.

*The deformed wing virus and the* Varroa destructor *mite are highly correlated. A synergetic relationship exists between both pathogens, which may predispose the colony to suffer losses. Application of acaricide treatments may be helpful for controlling viral replication.*

## SÉPTIMA / SEVENTH

La evaluación periódica de los niveles del virus de las alas deformadas y del ácaro *Varroa destructor* en las colmenas tiene un gran valor como sistema de seguimiento de la salud de las colmenas. Altas cargas de ambos patógenos pueden ser predictivas de problemas a nivel sanitario y se relacionan con un vigor más pobre de la colmena. Si los niveles se mantienen elevados durante al menos cuatro meses, aumenta el riesgo de colapso de la colmena.

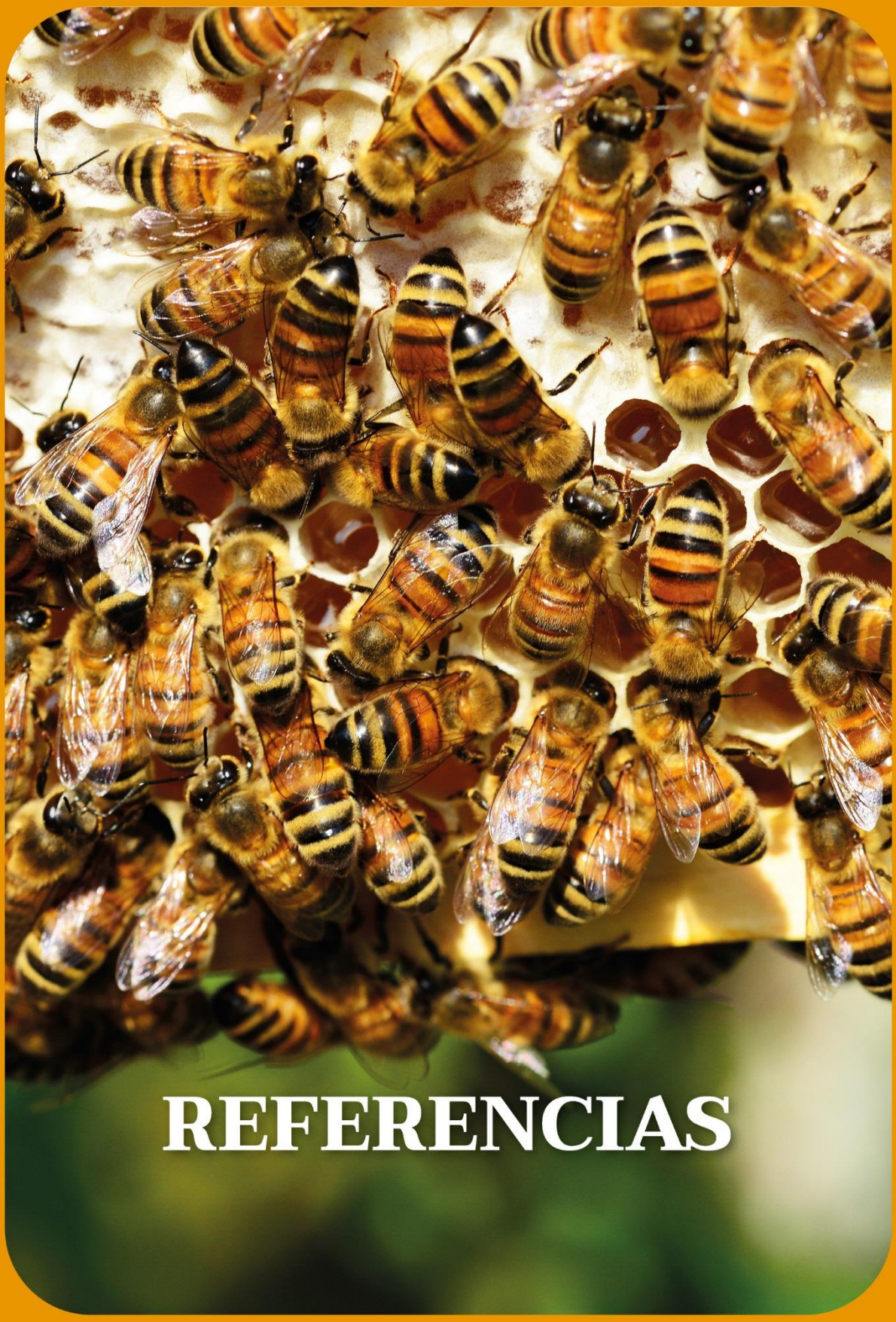
# CONCLUSIONES

*Monitoring deformed wing virus and* Varroa destructor *levels may be an effective tool for controlling colony health. High pathogen levels may predict health issues and reflect poor colony vigour. Four months of high levels may increase the risk of collapse.*

## OCTAVA / EIGHTH

La determinación del vigor de una colmena es un método eficiente, rápido y mínimamente invasivo para la evaluación de la fortaleza de la misma, siempre y cuando se realice por un apicultor o un técnico experimentado.

*Vigour determination is an effective, fast and minimally invasive method for colony strength determination, provided it is done by an experienced beekeeper or technic.*



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